AGENTS OF UNKNOWN CONCERN: ISOPROSTANES AS POTENTIAL EMERGING CONTAMINANTS

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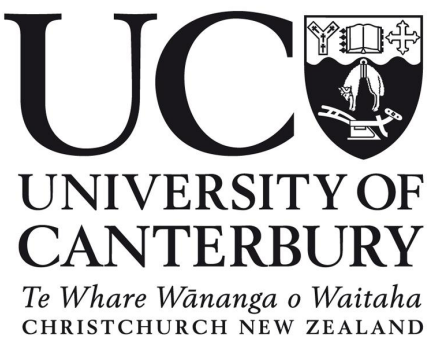

"We have come to know the distinct and undeniable impact that we as people have made and can make going forward on this one planet, we all must share."

- David Attenborough

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Acknowledgments

This thesis is dedicated to my mother, Hildy Kovacs.
Mamma, you are an inspiration and have taught me that hard work and commitment always pays off. Thank you for believing in me and encouraging me to pursue all that I am passionate about.

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Abstract

Wastewater treatment plants (WWTPs) are a primary source of many contaminants to the environment. Processing complex mixtures of waste, they can result in the continuous discharge of bioactive and endogenous compounds into sensitive aquatic ecosystems. Isoprostanes (IsoPs) are a class of potential emerging contaminants, produced in a range of vertebrate species, including humans, in response to oxidative stress. Released following exposure to various toxicants or disease, IsoPs are also mediators of inflammatory processes. Even in the low nanomolar range, these compounds have potent biological activities and can induce multiple pathophysiological responses, stimulating the continuation of oxidative stress. Their urinary excretion provides a mechanism for the entry of IsoPs to WWTPs and subsequently the wider environment, where they may initiate a cycle of oxidative stress in aquatic biota exposed to these compounds. Following which, additional IsoPs may be produced within these organisms, further perpetuating this cycle of toxicity.

To examine WWTPs as a source of IsoPs to the environment, an analytical method for their detection in wastewater based on solid phase extraction (SPE) and gas chromatography mass spectroscopy (GC-MS) was developed. Firstly, the novel method involved a deconjugation treatment with β-glucuronidase to increase the concentration of IsoPs available for detection. Subsequently, SPE followed by either silica gel chromatography or hexane liquid-liquid extraction were used to extract and purify the samples, removing the matrix inferences. Finally, a MSTFA derivatisation method was developed and resulted in the reliable detection of these compounds through GC-MS analysis.

A survey of Canterbury WWTPs was then conducted to assess the concentrations of IsoPs present in both wastewater influent and effluent. These compounds were detected at three of the four WWTPs assessed, with recovery-corrected concentrations ranging from ND-79.9 ng/L and ND-55.9 ng/L in the influent and effluent, respectively. As IsoPs were detected in wastewater effluent, this indicates that these compounds are not sufficiently removed during the treatment process, and that WWTPs may represent a source of these compounds to the environment. Further research is required to improve the extraction and purification methods in order to reliably assess the concentrations of IsoPs in wastewater. A series of comprehensive environmental surveys and ecotoxicological assays are also necessary to determine the potential roles of IsoPs as emerging contaminants.

List of abbreviations

15-F_{2t}-IsoP	15-F_{2t}-Isoprostane
BSTFA	N, O-Bis(trimethylsilyl)trifluoroacetamide
CDMTFS	Chlorodimethyl(3,3,3-trifluoropropyl)silane
C_{Cp}	Detected concentration of the surrogate in the comparative
C_s	Detected concentration of the surrogate
CVD	Cardiovascular disease
D_4-15-F_{2t}-IsoP	15-F_{2t}-isoprostane-D_4
DEA	Diethylamine
DI	Deionised water
DIMETRIS	Dimethyl(3,3,3-trifluoropropyl)silyldiethylamine
DMTFPS	Dimethyl(3,3,3-trifluoropropyl)silyl
EC	Emerging contaminant
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
En	Enzyme
FLPR	Strata Florisil pesticide grade cartridge
GC-MS	Gas chromatography mass spectroscopy
HPLC	High pressure liquid chromatography
IQL	Instrument quantification limit
IsoP	Isoprostane
LOD	Lower detection limit
MDL	Method detection limit
MQ	Ultra-pure water
MSTFA	N-Trimethylsilyl-N-methyl trifluoroacetamide
N/A	Not applicable
NCI	Negative chemical ionisation
ND	Not detected
PFB-Br	Pentafluorobenzyl bromide
PTFE	Polytetrafluoroethylene
R%	Recovery (%)
R_c	Recovery corrected concentration

RIA	Radioimmunoassay
ROS	Reactive oxygen species
S	Spiked sample
SIM	Selected ionisation mode
SPE	Solid phase extraction
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TP	Prostanoid receptor
W	Wastewater
WD	Weekday wastewater samples
WE	Weekend wastewater samples
WWTP	Wastewater treatment plant

Chapter 1

Introduction and research objectives

1.1 Wastewater treatment plants, emerging contaminants and environmental pollution

Wastewater treatment plants (WWTPs) are a primary source of many anthropogenic pollutants entering the environment.[1-3] Wastewater overflow, the release of effluent and the application of sewage sludge as a soil amendment each contribute to their total discharge.[4, 5] During 2018, there were forty wastewater overflow events in Christchurch, New Zealand. Each incident resulted in the discharge of untreated wastewater to the environment and heralded the potential for contamination to remain for up to two days once the source had stopped.[6] On average, sewage overflows across the country related to wet weather increased by 379%, compared to the previous year.[7] Growing populations, aging infrastructure, and an increase in severe weather events due to climate change are all factors which place a strain on our wastewater systems and infrastructure. Each factor compromises the transport of wastewater to the treatment plants, while also contributing to an overall decrease in plant function and treatment efficiency, potentially resulting in the purposeful or accidental discharge of untreated wastewater to the environment. These issues, combined with the release of properly treated wastewater effluent, accounts for a burgeoning issue worldwide.

Complex mixtures of chemical contaminants from a combination of municipal, industrial and agricultural waste inputs are processed in WWTPs.[1, 4, 5] Although WWTPs remove many contaminants from waste, inadequate treatment methods or the transformation of compounds can result in their incomplete removal, especially in developing countries.[4, 8 9, 10] Of particular concern are emerging contaminants (ECs), which are defined as newly-synthesised compounds and those which have only recently been detected or identified as being contaminants of concern. In conjunction with advancing technology, population growth, and changing socioeconomic classes, the list of potential ECs is increasing.[1,10] Compounds which previously have not been of concern, may now pose risks due to the sheer volume produced and discharged. Many of these compounds exert ecotoxicological impacts in their receiving

environments, especially on vulnerable aquatic species.[9] In many countries, such as New Zealand, important species (e.g. native or food source) are already in decline[11] and additional pressures from exposure to contaminants have the potential to push these ecosystems past their tipping points.[1]

1.1.1 Synthetic emerging contaminants

Synthetic ECs, including pharmaceuticals and personal care products (PPCPs), pesticides and microplastics are of increasing environmental concern.[9, 12] Many of these compounds are designed specifically to ensure their long-term chemical stability or potent biological function. In some instances, even if a compound has not been developed for these purposes, it may still possess certain undesirable biological activities due to structural similarities to natural biological compounds.[1] For example, various pesticides, industrial compounds, and PPCP preservatives possess endocrine disrupting properties as a result of their structural similarities to steroid hormones.[1, 10] There is concern over the presence of these compounds in the environment as they do not exist in isolation, but as diverse mixtures, which could result in complex and adverse synergistic effects.[4] Additionally, even if a parent compound is perceived as harmless, its metabolites may be more persistent, with similar or more harmful ecotoxicological impacts. Both parent and daughter compounds can have complicated and poorly-understood environmental characteristics and fates, making the regulation, management, or mitigation of these contaminants difficult.[9, 13, 14] Even if one is removed from the market, another may easily take its place with an even less understood toxicity. Warranted concern over the threats that these poorly-understood compounds pose has resulted in increased research and the intensification of existing contaminant sampling programmes.[12]

1.1.2 Endogenous emerging contaminants

Biological pollutants make up another category of micropollutants likely to become of increased importance as ECs.[12] These include pathogenic viruses and bacteria, and endogenous compounds. The latter may be defined as those which originate from within an organism or cell.[15] Endogenous compounds may be produced in response to natural or abnormal biological processes, and they often have additional roles in mediating biochemical processes.[15, 16] Changes in physiological state are associated with fluctuations in their concentration and rate of production, and while these changes can be linked to regular functions and metabolism, they can also be the result of exposure to external toxicants or disease.[17] The synthesis of

endogenous compounds can result in the propagation of pathological responses due to their biological roles, especially if they are produced as a result of an abnormal or aggravated biological state. Bioactive endogenous compounds which are excreted by humans are of particular concern as potential ECs.[1, 18] Steroid hormones and endocrine disrupting chemicals, such as estrogenic compounds and glucocorticoids, are examples of ECs which have been detected throughout environmental matrices. These compounds are capable of eliciting adverse effects at low but environmentally relevant concentrations and their impacts have been widely assessed.[1, 19, 20] WWTPs contribute significantly to the discharge of steroid hormones, as most plants are not completely equipped to remove these contaminants excreted by humans. As a result, aquatic species living downstream of these sources are often the most exposed and severely affected by the contaminants contained in the effluent.[21]

Our understanding of the biological activities of many endogenous compounds continues to develop. Despite this knowledge and our understanding of the conservation of metabolic processes between species,[22] limited research has been conducted to investigate other non-hormone compounds in terms of their potential roles as ECs. It is likely that humans or other mammal species are excreting compounds capable of eliciting pathological responses into WWTPs. These may then be discharged to the wider environment, where they could cause further harm to organisms in the receiving ecosystems. Many bioactive compounds have the potential to act as contaminants and impact species within an ecosystem if released at high enough concentrations.[2] Further research into different potential contaminants is required, to enable the quantification and mitigation of the harm caused by these unknowns. Although only low levels may be excreted or released into wastewater, the consequences of exposure to endocrine disrupting compounds have demonstrated that species can still be severely impacted, leading to increasing concern over the potential harm that these relatively unstudied contaminants may be causing in the environment.

1.1.3 Factors which may contribute to the concentrations of endogenous compounds in wastewater

Although WWTPs are responsible for the removal of many contaminants from wastewater, inadequate treatment methods or the transformation of chemicals can result in their incomplete removal.[4, 8] Increasing population density, substance abuse, and the continuous discharge of effluent may exacerbate the impacts of environmentally concerning concentrations of contaminants released into sensitive environments. Research is still required to determine

effective mechanisms to isolate, detect and manage these compounds.[8, 23] Once released into the environment, despite rapid degradation rates, the continuous input of these compounds from WWTPs can result in pseudo-persistence and a long-term environmental presence.[9] The concentrations of endogenous compounds discharged from a particular WWTP will vary based on the size of the population being served, the industries it is serving, and the population's overall health.[17] For many endogenous compounds, their production and excretion are dependent on organism health. It is expected that in lower socioeconomic areas where medical treatment may be limited, or areas where substance abuse is more prevalent, higher concentrations may be present in wastewater.[17]

1.2 Wastewater epidemiology biomarkers as potential emerging contaminants

1.2.1 Endogenous compounds as sewage biomarkers to assess community health

The production and excretion of various endogenous compounds within an organism are largely dependent on its health status.[24] Therefore, analysis of faeces or urine for a specific compound or biomolecule can provide a non-invasive method to assess health, based on an understanding of how biological state dictates its production. This concept is being exploited for wastewater epidemiology, where the measurement of endogenous or exogenous compounds excreted by humans can be used as a tool to assess community health.[16, 17, 25] Originally proposed to evaluate substance abuse, wastewater epidemiology has been extended further, through to understanding community exposure to various ECs and assessing general health or disease status.[17, 25, 26] The latter is performed either directly, by searching for endogenous inflammatory biomarkers (produced in response to disease or inflammatory processes), or indirectly, through analysing for pharmaceutical drugs which provides an idea of their consumption and the proportion of the population that require them.[25]

Many different endogenous compounds have been proposed as potential health biomarkers because of their relationship or production in response to common diseases (such as atherosclerosis, diabetes, and cancer), general ill-health, and physiological dysfunction or stress.[25] Understanding the physiological factors which result in the production of a biomarker, as well as individual rates of excretion, are important for its success. Then, through analysing

the concentration of a "successful biomarker" in influent, an understanding of the overall health of a population can be determined.[25] A subset of the currently proposed sewage biomarkers also have known or suspected roles in the pathogenesis of health issues in humans.[17] Whereby, elevated concentrations of pro-inflammatory molecules have been linked to the exacerbation of various diseased states, including inflammation, cell proliferation, cell death, and many other biological processes. Table 1-1 provides a brief summary of proposed epidemiology markers which have additional known or suspected biological roles. Although only isoprostanes (IsoPs) have begun to be investigated for these roles, some of these other molecules, alongside IsoPs, may represent potential contaminants, justified by their urinary excretion and biological activities (Section 1.2.3).

Table 1-1. Proposed wastewater epidemiology biomarkers and limitations to their understanding as emerging contaminants.

Biomarkers	Biomarker description	Relationship to disease	Urinary excretion	Limitation
CRP	- An acute-phase protein involved in inflammation. - It may be used as a biomarker of oxidative stress in humans.[27]	- Can be detected in plasma in response to cancers, cardiovascular disease (CVD), urinary tract infections, pneumonia, appendicitis and meningitis.[27-29] - Elevated levels are linked to the potential pathogenesis of disease.[30]	- Yes. - It can be detected at elevated levels in urine in response to disease.	- More information relating to its urinary excretion and relationship to the propagation of disease is required.
Nitro or chloro-tyrosine	- They are metabolites of tyrosine, produced by the action of myeloperoxidase (MPO), an oxidising enzyme involved in inflammation.[31,32] - 3-chlorotyrosine and nitrotyrosine serve as clinical biomarkers of MPO activity.[31, 32]	- 3-chlorotyrosine causes endothelial dysfunction, increases free-radical production, and is linked to atherosclerosis.[33] - High levels are found in patients with coronary artery heart disease and asthma. - Nitrotyrosine levels are elevated in diabetes and influenza patients and smokers.[33] - Protein nitrotyrosine formation may be a risk factor for CVD.[34] - Believed to have a role in atherosclerosis.[34]	- Yes. - Elevated levels can be detected in urine in relation to multiple pathological conditions in both humans and other animals.	- Halogenated compounds may be released from food and other sources into sewage.[17] - Nothing is known about their fate or concentrations in sewage. - It is not known if exposure results in the propagation of inflammation or oxidative stress symptoms.
***o,o'*-Dityrosine**	- *o,o'*-Dityrosine forms when a radical (e.g. hydroxyl) crosslinks tyrosine residues.[35]	- Increased plasma concentrations are detected as a result of oxidative stress in children.[31] - Increased concentrations are linked to atherosclerosis and aging.[36]	- Yes. - Elevated levels can be detected in urine in response to disease.	- Very little is known about its fate in urine. - Nothing is known of its fate in sewage.
Isoprostanes	- A group of compounds which are produced in response to the peroxidation and cyclisation of fatty acids. - They are used as clinical biomarkers of oxidative stress.	- They are formed in response to, and have roles in a range of acute and chronic inflammatory diseases. - Patients suffering from diseases such as hypertension, diabetes, obesity, neurodegenerative disease and asthma have elevated urinary and plasma levels.	- Yes. - They can be detected at elevated levels in urine in response to disease.	- Limited information on their behaviour in sewage is known.

1.2.2 Wastewater epidemiology and emerging contaminant interface

In a wastewater epidemiology context, the bioactivity of a compound is not of primary importance. However, wastewater epidemiology does intersect with EC research.[16] From an EC perspective, any compounds released into wastewater at high enough concentrations, with additional biological roles, may represent potential contaminants. A holistic approach can be applied to the two fields, as certain characteristics of potential wastewater biomarkers match the characteristics of potential contaminants, including: (I) Excretion in the urine or faeces in constant or consistent concentrations; (II) Present in wastewater at detectable concentrations; and III) Chemically stable in wastewater.[17, 24, 37] Although these compounds may not have negative biological effects in the originating species or organism, other species may react adversely in response to exposure. Moreover, estrogens make a pertinent example. Natural forms of these hormones are important for biological and physiological function throughout organisms.[38] Females of child-bearing age naturally have greater concentrations of estrogenic compounds compared to individuals in different life-stages (e.g. children or post-menopausal women), sexes, or species. These same elevated concentrations are not necessary for biological functions in latter groups of individuals, and exposure to additional xenoestrogens has the potential to induce adverse effects.[39] These include reduced sperm counts, premature puberty, lower fertility rates, and other forms of altered physiology.[1] So although females may be exposed to high concentrations of estrogens through their natural production and from birth control pills, what they excrete into sewage can have a continued and unwanted secondary impact on other individuals or species.

Population growth will likely increase the concentration of endogenous compounds present in wastewater,[16] both improving their detection in epidemiology studies and increasing the risk they pose to the environment. As previously mentioned, more research investigating other endogenous compounds as potential ECs is required.[40] Epidemiological research on potential biomarkers has provided a useful resource and perspective on many different compounds (Section 1.2.1), which may otherwise not have been considered as potential contaminants. Whether or not they prove detrimental to the environment, or are excreted at great enough concentrations to pose a threat, wastewater epidemiology provides a platform upon which further investigations can be conducted.

1.3 Isoprostanes: Clinical and epidemiological biomarkers of oxidative stress

1.3.1 An introduction to isoprostanes

Isoprostanes are a group of bioactive, clinical biomarkers produced in response to oxidative stress in many vertebrate species, including humans.[41-43] These prostaglandin-like compounds are formed *in vivo* through the free radical-catalysed peroxidation of membrane-bound, esterified fatty acids (Section 1.3.2).[44, 45] In the blood of healthy individuals, IsoPs are found at nanomolar concentrations which can then increase by several orders of magnitude following an oxidative stress event or when the body is in a diseased state (e.g. vascular or pulmonary disease).[41, 46, 47] IsoPs are formed throughout the body and as a result they have been detected in a range of biological matrices, including amniotic fluid,[48] cerebrospinal fluid,[49] atherosclerotic plaques,[50] exhaled breath condensate,[51] plasma,[52] and urine.[53] Coupled with their abundant formation in response to oxidative stress and oxidative stress-linked diseases, this has led to the use of IsoPs as clinical biomarkers of these compromised physiological states (Section 1.3.4). The elevated concentrations of these compounds in plasma and urine provides an advantageous method to determine oxidative stress status and disease progression in many species. Additionally, their presence in urine has resulted in their use as wastewater epidemiology biomarkers (1.4.2). Which furthermore, has led to the consideration of their potential role as ECs, as proposed in the work of Gaw and Glover (2016)[18] and subsequently, Pais et al. (2018).[54] The theory behind the roles of IsoPs as ECs capable of participating in feedback loops of toxicity is further explained below (Section 1.5).

1.3.2 Biosynthesis of isoprostanes

The biosynthetic pathways of IsoPs proceed through several radical steps and provide a mechanism for the formation of multiple classes of IsoPs, which differ based on the functional groups on the prostane ring.[55, 56] While the following pathway (Figure 1-1) describes the formation of F_2-IsoPs, D_2/E_2 IsoPs can also be produced through rearrangement of the endoperoxide intermediates. The D_2/E_2 IsoPs are formed in competition to the F_2 series, especially when under depletion of reducing agents (e.g. a-tocopherol and ascorbic acid).[55]

Following an oxidative stress injury which results in the release of free radicals in the cellular membranes, the generation of F_2-IsoPs is initiated by the abstraction of a bis-allylic hydrogen

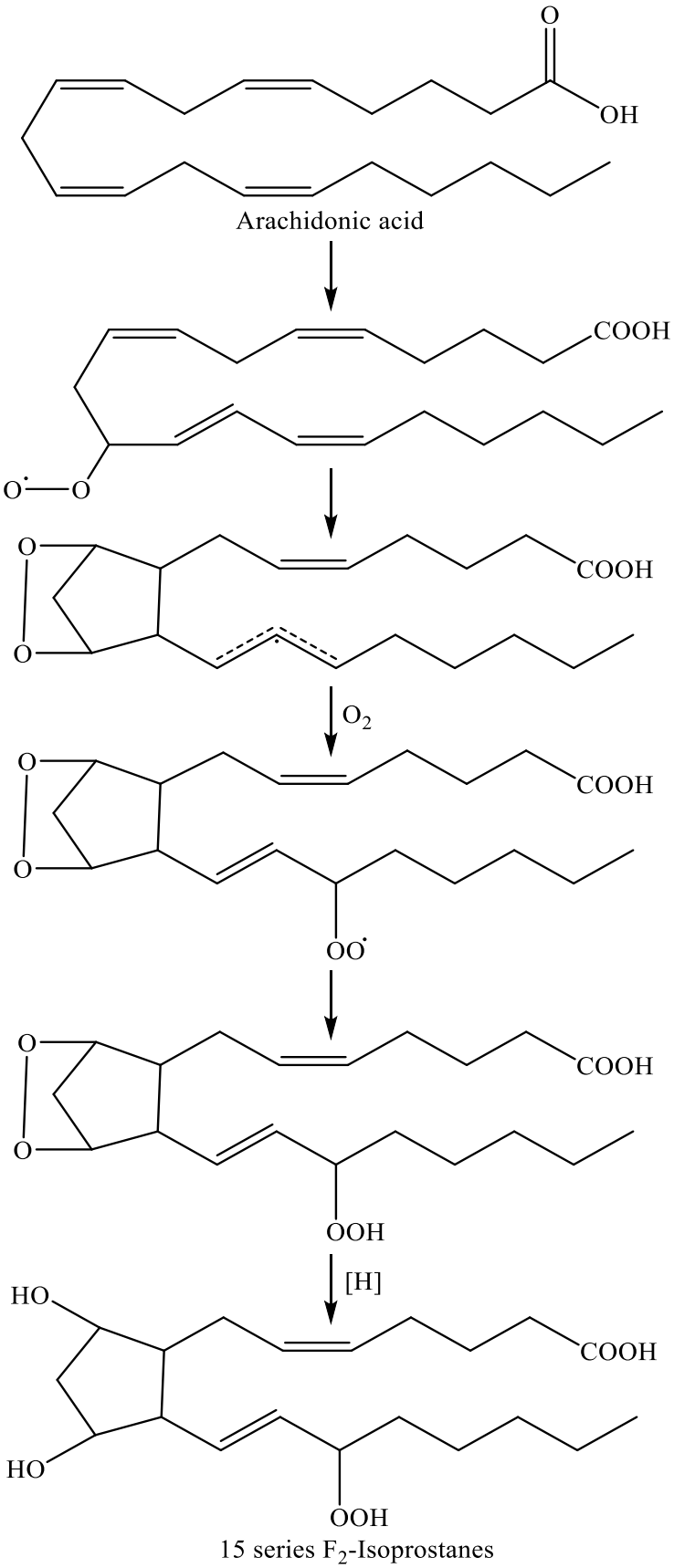
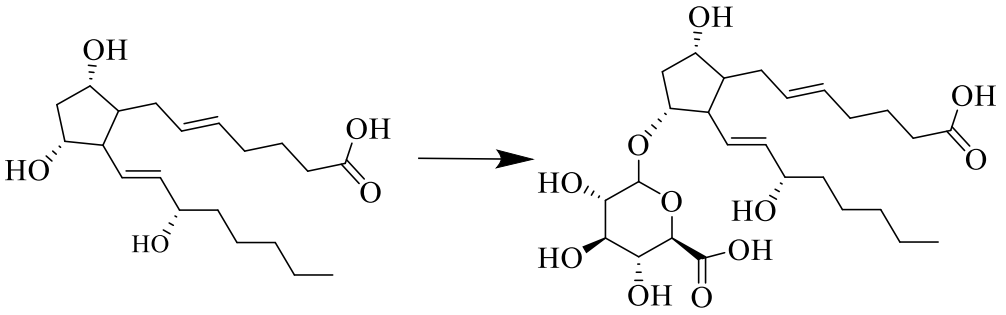


Figure 1-1. Mechanism of formation for the 15 series F_2-IsoPs from the free radical- initiated peroxidation of arachidonic acid.

atom from arachidonic acid by a radical species.[55] Subsequently, the radical reacts with a molecule of oxygen (O_2), resulting in the formation of a peroxyl radical which then undergoes 5-exo cyclisation before it reacts with a second oxygen molecule. A final reductive sequence of the endoperoxide and hydroperoxide functionalities leads to the formation of four regioisomeric F_2-IsoPs, dependent on the site of hydrogen abstraction and oxygen insertion. For each of the four regioisomers, there are 8 racemic diastereoisomers. Although there may be 64 possible compounds generated, some predominate over others, such as the 5 and 15 F_2-IsoP series which are formed in greater proportions compared to the 8- and 12-series as they do not undergo further oxidation.[55, 56, 57]

Isoprostane formation predominantly occurs in esterified form. Most of the precursor fatty acids are membrane bound, and exist as phospholipid esters in the interior of cell membranes.[44] Phospholipase action releases the IsoP molecules, whereby they circulate in the plasma and can be excreted in the urine in free form or as glucuronic acid conjugates (Figure 1-2). Once in circulation, these compounds can also undergo metabolism to form additional metabolites. 15-F_{2t}-IsoP, for example, is β-oxidised to form the intermediate compound 2,3-dinor-15-F_{2t}-IsoP, which further reduces to 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP.[58, 59]



15-F_{2t}-Isoprostane Glucuronide conjugate of 15-F_{2t}-IsoP

Figure 1-2. Glucuronidation reaction of 15-F_{2t}-IsoP, one of the more commonly assessed IsoP isomers.

1.3.3 Physiological roles and consequences of free radicals and reactive oxygen species

Free radicals are defined as highly reactive chemical species which contain one or more unpaired electrons in their outer orbitals.[55] In comparison, reactive oxygen species (ROS) are also chemically reactive compounds, however these are specifically oxygen-containing molecules. These include oxygen-centred radicals, oxygen ions and peroxides. Hydrogen

peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$), alkoxyl radicals ($RO^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$) are the most common ROS generated in living organisms. Other reactive species include nitric oxide (NO), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and other carbon-centred radicals.[55] ROS produced *in vivo* are derived from both endogenous processes (e.g. primarily metabolic and respiratory processes) and exposure to exogenous toxins (e.g. environmental pollutants, radiation injury, lifestyle toxins, etc).[60] Redox reactions occurring during aerobic metabolism also result in the continuous generation of ROS.[61] Mitochondria in particular, are a major source of these endogenous ROS in cells. While producing energy, the electron transport chain releases small amounts of superoxide due to the 'leaking' of electrons directly to oxygen.[62] An estimated 1-4% of oxygen reacting with the electron transport train is incompletely reduced to the superoxide anion by these 'leaking' electrons. Under conditions of cellular stress and ATP depletion an excess of superoxide and H_2O_2 causes the release of free iron (Fe^{II}) from iron-containing molecules, such as iron-sulfur proteins within the mitochondrial membrane. This promotes the formation of the highly reactive hydroxyl radical, which can cause further damage.[55]

ROS production also plays an important role in immune defence. Phagocytes are activated in response to inflammatory mediators and foreign micro-organisms. Then, nicotine adenine dinucleotide phosphate oxidase (NADPH NOX) produces superoxide anions catalysing the transfer of electrons from NADPH to molecular oxygen to form superoxide.[63] Additionally, myeloperoxidase, a heme-containing phagocytic enzyme, produces hypochlorous acid (HOCl) in neutrophils, through reactions with hydrogen peroxide and chloride at sites of inflammation.[64] However, the production of ROS at these sites of inflammation can also cause further injury to surrounding tissues, compounding the state of oxidative stress.

Often abnormal increases in oxidative stress within organisms, via endogenous processes or pro-oxidant exposure, is mitigated by antioxidant response. The balance between pro-oxidants and anti-oxidants is critical in maintaining the proper redox environment within cells for their health and survival.[65] It can easily be disturbed by an overproduction of ROS or insufficient antioxidant defence mechanisms, resulting in oxidative stress.[66] Under these conditions, high levels of ROS react non-specifically and rapidly with cellular biomolecules, resulting in DNA mutations, protein oxidation and lipid peroxidation.[62] The oxidation of proteins impacts their activity and function. The overproduction of lipid peroxidation products has been linked to the pathogenesis of several diseases. Cellular activity and function is disrupted via oxidative

damage to cell membranes and the disturbance of membrane organisation, integrity, fluidity and permeability. In general, oxidative stress is increasingly being implicated in the aetiology of many human diseases.[67, 68] These include autoimmune disorders, cancer, CVD, diabetes and neurodegenerative diseases.[69] Therefore, many biomolecules with the ability or function to increase oxidative stress have also been implicated with the pathogenesis or progression of many diseases (e.g. aging, atherosclerosis, and neurodegenerative disease).[61] Consequently, IsoP production is also linked to many of these diseases and processes (Section 1.3.4).

1.3.4 Isoprostanes as biomarkers and mediators of oxidative stress

Biomarkers

The relationship between oxidative stress and (I) the production of IsoPs; and (II) the onset of disease; makes these compounds useful biomarkers. As mentioned previously (Section 1.3.3), oxidative stress is the result of an imbalance in biological redox status. A variety of stressors can disrupt this balance, driving it in favour of oxidants.[55] The prevalence of physiological oxidative stress can disrupt redox signalling and damage biomolecules which can lead to the onset of disease, linking oxidative stress to a variety of chronic and noncommunicable diseases. IsoPs are used as clinical biomarkers of oxidative stress in humans.[43] Many isomers, including 15-F_{2t}-Isop, the F_2, D_2, and E_2 series, can be reliably detected in biological fluids (e.g. plasma and urine) at elevated concentrations in response to various stressors.[43, 58, 70] Disease, lifestyle toxicants, and natural biological processes can all result in an increase in total IsoP production due to an increase in ROS and oxidative stress. Additional factors, such as exercise and diet may also contribute to their physiological concentrations and production.

Generally, the concentrations of free 15-F_{2t}-IsoP is greater in the urine (100-1200 ng/L) compared to plasma (0.035-45.1 ng/L).[71] Coupled to the non-invasive nature of sample collection, urine provides a useful method to assess oxidative stress, disease status, and each of their relationships to exposure to environmental toxicants. Many diseases have shown associations with elevated levels of 15-F_{2t}-IsoP. Of these, some of the more notable include: Alzheimer's, congestive heart failure, cystic fibrosis, cancer (such as breast, prostate, and stomach), alcoholic liver disease, chronic renal insufficiency, obesity and diabetes, among others.[72] However, a meta-analysis by van Erve et al. (2017)[71] identified that the association between IsoP concentrations and disease-type differs from previously predicted values, with

Alzheimer's, cancer, and obesity showing lower associations compared to many others despite their significant links to oxidative stress.[71]

Mediators of biological processes and pathways

IsoPs are not simply diagnostic by-products of oxidative stress. They have complex biological activities, whereby they can mediate many different inflammatory processes.[45, 73, 74, 75] Of the cocktail of IsoP isomers produced, many are involved (both directly and indirectly) in natural and pathophysiology.[76] These compounds initiate biological pathways through interactions with various biomolecules. Although, the entirety of biomolecules which they can interact with has yet to be fully determined. Based on research so far, it is theorised that IsoPs contribute to the functional and pathophysiological consequences of oxidative stress primarily through the interaction and the activation of the thromboxane A_2 (T_XA_2) prostanoid receptor.[45] Additional activities may also be mediated through interactions with a receptor unique to IsoPs[45] or through other prostanoid receptors. The latter is unsurprising due to their structural similarities of IsoPs to prostanoid molecules.

Thromboxane A_2 is a highly reactive and short-lived molecule involved in many processes through interactions with the prostanoid receptor. These processes include angiogenesis, atherogenesis, hypertension, myocardial infarction, vasospasm, and thrombosis.[77, 78] Because many IsoP isomers are able to interact with this receptor, elevated concentrations of IsoPs have been linked to many of these processes as well. Studies have found that the administration of IsoPs (e.g. 15-F_{2t}-IsoP and 8-Iso-prostaglandin E2) can result in the significant increase of blood pressure in rats.[79] However, these hypertensive effects are not observed in prostanoid receptor-deficient organisms and can also be prevented following the administration of prostanoid receptor antagonists.[79, 80] Alongside these vasomotor effects, IsoPs have proatherogenic properties, contributing to the initiation, progression, and physiological complications of CVD.[75, 81] Usually, T_XA_2 and prostanoid receptors play important roles in CVD progression, with roles regulating platelet activity, endothelial integrity, and leukocyte-endothelial cell interaction.[82] Based on the evidence, prostanoid receptor agonists other than T_XA_2, such as IsoPs, may be important in regulating the initiation and progression of atherosclerosis. Especially given what has already been deduced surrounding IsoP-prostanoid receptor interactions.

Alongside these vasomotor effects, IsoPs can also lead to DNA synthesis and endothelial cell proliferation.[83, 84] The role these effects have on the aetiology of pathophysiological conditions and disease processes has yet to be fully established. Although, it is theorised that these effects will also allow IsoPs to contribute to CVD and other diseases through inducing enhanced interactions between endothelial and immune cells, such as the monocytes and neutrophils.[85] In this case, likely through modulating endothelial cell-immune cell interactions through both prostanoid receptor dependent and independent mechanisms.[86]

Studies have found that another mechanism through which IsoPs may be indirectly altering physiological state is by inducing prostaglandin formation. Basu (2006)[87] suggests that exposure to pharmacologically-administered or endogenously-produced 15-F_{2t}-IsoP, during an oxidative stress event, induces prostaglandin formation. Most probably, this occurs through the classic cyclooxygenase-catalysed arachidonic acid oxide ion reaction. This may have an important biological role in the pathogenesis of free radical-related diseases and the initiation of inflammation, as prostaglandins themselves have important roles in controlling various signalling pathways.[87] These include balancing physiological (renal, vascular, parturition, labour induction) and pathological (vasoconstriction in diseased states and inflammation) status. If IsoPs are capable of directly impacting the availability of prostaglandins in the biological system, this may cause additional physiological (and pathophysiological) consequences, based on how different prostaglandin concentrations usually modulate these processes. These impacts may include contributions to acute inflammatory response or the progression of chronic inflammation.[60] Therefore, in a situation where there is a sudden increase in IsoP concentrations, irrespective of the stimuli (e.g. acute or chronic oxidative stress, or exogenous administration of these compounds to the body), these physiological consequences may still be brought about, resulting in a cycle of oxidative stress due the initiation of F_2-IsoP biosynthesis. This highlights an additional link between elevated IsoP concentrations and their subsequent roles in free radical-related disease, the initiation of inflammatory response and oxidative stress.[60, 75]

Isoprostanes induce a diverse range of biological activities in the low nanomolar range, with many cell types being found to respond in a pathologically-relevant manner, following exposure to the molecules (Figure 1-3)[74, 84, 88, 89] These effects have primarily been researched and documented in rodents and humans. However, due to the production of IsoPs throughout

mammalian and vertebrate species and the conservation of metabolic processes, similar mechanisms of action would be expected throughout species and cells. The biological effects are induced throughout IsoP classes, isomers and some metabolites, such as 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP. Involved in a variety of pathological conditions, even if they are not direct causal agents of disease, IsoPs are still agents capable of exacerbating such clinical conditions.[90] Additionally, this mounting evidence suggests that they can cause the continuation of a cycle of oxidative stress following the initial event. IsoPs are also thought to be capable of acting synergistically, which can result in a response which is greater than the sum of individual IsoP effects,[91] an effect analogous to the well-documented synergistic activity of xenoestrogens.[92, 93] This is significant as many of the isomers have different biological potencies or activities. Therefore, based on the cocktail of compounds and biomolecules present and the cell type, a complex range of responses may be elicited by the IsoPs.[90] Which raises the question, that should exogenous exposure to a cocktail of such compounds occur, would this result in the propagation of disease processes and further IsoP production?

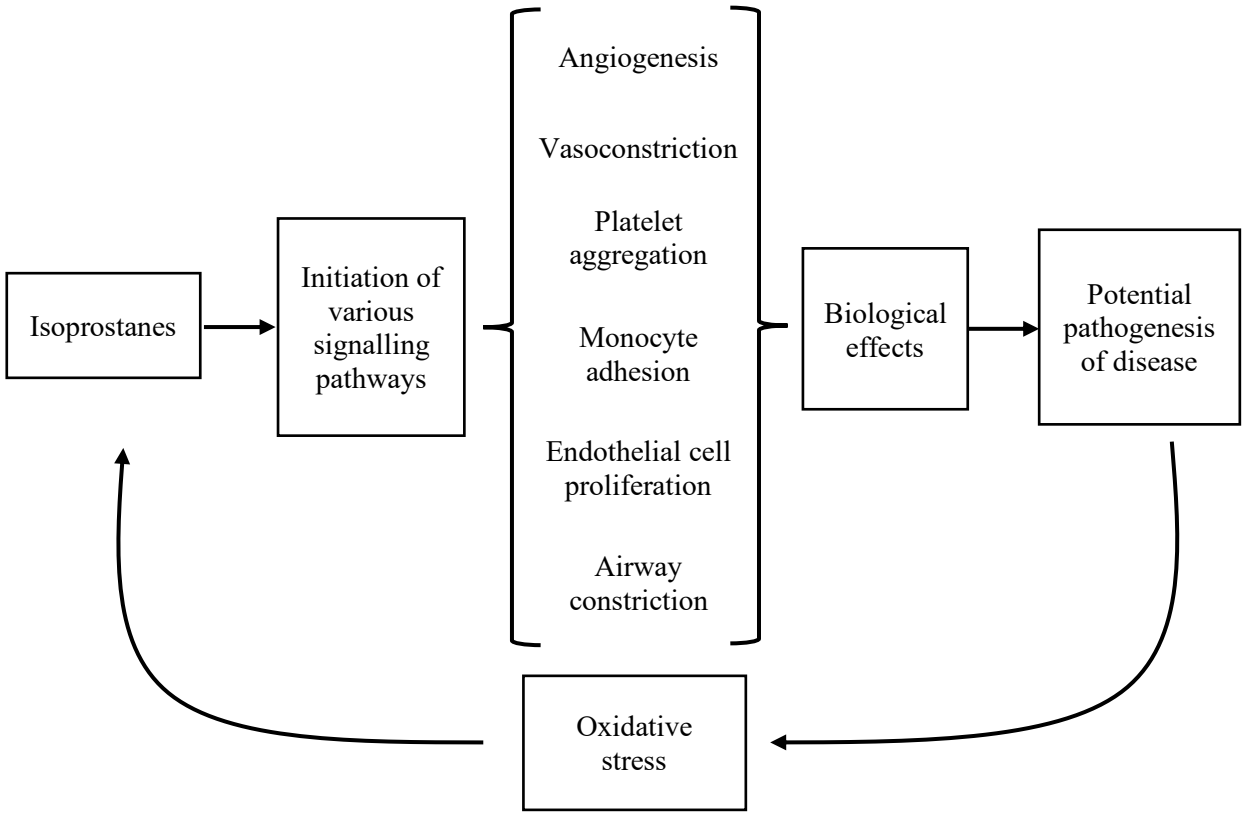


Figure 1-3. A scheme of various isoprostane-initiated physiological pathways, and their subsequent biological activities which can lead to the induction of additional oxidative stress.[75, 86]

1.4 Methods for the quantification of isoprostanes in biological samples and wastewater for their roles as epidemiology biomarkers

1.4.1 Methods of detection

The methods currently available for IsoP quantification are primarily based on chromatogram separation with mass spectrometry detection (e.g. GC-MS and LC-MS) or immunological methods.[91] Due to their relatively high chemical stability, chromatographic and mass spectrometric characteristics, GC-MS in negative chemical ionisation (NCI) mode has been shown to be a reliable analytical technique to assess these compounds.[94] Following extraction, F_2-IsoPs are most commonly derivatised to form pentafluoryl esters and trimethylsilyl ether (PFB-TMS) derivatives. Through this method, the pentafluorobenzyl (PFB) group esterifies to the carboxyl group of the IsoP, while the TMS groups derivatise the remaining hydroxyls, which allows the detection of a single, intense ion m/z 569.[58, 70, 94, 95] To further improve this method, some researchers have developed tandem mass spectrometric methods. One of the biggest limitations to the use of PFB-TMS is the inability to distinguish between different F_2-IsoP compounds.

High performance liquid chromatography mass spectrometry (HPLC-MS), with electrospray ionisation, is another selective and sensitive method considered comparable to GC-MS, with the advantage that sample preparation is simplified without the need for derivatisation.[96] Additionally, this method can enable differentiation between the F_2-IsoP regioisomers, as unique ions can be identified in the lower m/z region dependent on the compound (e.g. 5-F_2-IsoP m/z 115, 15-F_2-IsoP m/z 193, 8-F_2-IsoP m/z 127, 12-F_2-IsoP m/z 151).[97] The development of these methods allowed 5-F_2-IsoP and 5-epi-5-F_2-IsoP to be determined as the most abundant IsoPs in human urine, despite 15-F_{2t}-IsoP being the compound which is most commonly investigated and analysed in literature.[96, 98]

Finally, while these mass spectrometry methods are useful due to their sensitivity and accuracy, they are not always accessible for all researchers and can be time consuming to conduct.[96] To overcome these issues, commercially available radioimmunoassays (RIA) and enzyme immunoassays (EIA) have been designed.[96] An example is the ELISA (enzyme-linked immunosorbent assay) kit. Although they are more accessible to researchers, they are readily compromised by the presence of interfering substances found in more complex biological

matrices or fluids. Extensive purification methods such as HPLC or thin layer chromatography (TLC) used alongside these kits can help under such circumstances, however if only partial purification occurs, the interfering substances can end up being concentrated down.[96] Additionally, the concentrations detected from EIA methods compared to others are not always comparable with mass spectrometric methods. This can be due to the EIA methods not always being specific to individual isomers, which can skew the results if there are multiple present within a sample. EIA methods can also be extremely biased, and this cross-reactivity can overestimate the concentrations of IsoPs present in a sample, even when there is a correlation between their results and those found through mass spectrometric methods.[99] As such, clinical results achieved using spectrometry versus EIA should not be compared.[76,96]

Extraction and purification procedures are necessary to optimise detection when handling biological samples, despite them being time-consuming and often leading to a loss of analyte. Solid phase extraction (SPE) occasionally followed by additional TLC purification is one of the most common IsoP isolation techniques. A multitude of SPE methods and cartridges have been trialled with IsoPs.[70, 96] The more common cartridges include Oasis HLB,[100] Oasis MAX, Strata X,[101] Strata X-AW[100] and reverse phase (C_{18}),[100, 102] some of which achieve recoveries ranging from 50-100% with relatively good reproducibility.[100, 103] Of the extraction techniques there is little consistency in the properties of the molecules which are being targeted, as base-, neutral- and acidic- selective cartridges have all been used to varying degrees of success. Immunoaffinity chromatography (IAC) has also been used for IsoP extraction.[42, 104] This technique effectively extracts the IsoPs without the need for the SPE or TLC steps, following which the sample can then be prepared for analysis. This reduction in steps is often useful as it reduces sample handling which can otherwise introduce the potential for loss in analyte or contamination.[104] Commercially available columns have made this method more accessible to laboratories, but they are still relatively expensive.

Deconjugation is an additional stage that can provide improved detection of IsoPs in urine samples.[105] Glucuronide conjugates are formed through the enzyme-catalysed glucuronidation of compounds that contain either phenol, hydroxyl or carboxyl groups, yielding a more water-soluble derivative for excretion from the body. As noted (Section 1.3.2, Figure 1-2), IsoPs are excreted from the body as these conjugates. It has been demonstrated through treatment of urine with β-glucuronidase, that a 40% increase in the concentrations of F_2-IsoPs detected can be achieved.[105] Additionally, given that some of these compounds are biologically active, the

formation of these IsoP-glucuronides may be important in moderating their biological activities.

1.4.2 Isoprostanes as wastewater epidemiology markers

As mentioned previously, various exogenous and endogenous compounds produced by humans have been proposed as potential wastewater epidemiology biomarkers (Section 1.2). Two groups (Ryu et al.[106] and Santos et al.[107, 108]) have recently conducted pilot studies investigating the roles of IsoPs as wastewater-derived biomarkers of community health. In the first studies by Santos et al. (2015 and 2016),[107, 108] the group used a commercially available ELISA kit (Detroit R&D, Inc) to quantify the concentrations of IsoPs in influent (1-16 ng/L)[107, 108] and effluent (not detected).[108] The samples were collected from three collection points from May to June 2014[107] and over a one year period from May 2014 to June 2015.[108] While the trends in concentrations were found to be consistent over time, they did vary between sites. The two collection sites which served larger and more urbanised populations had greater IsoP concentrations compared to the smaller site, meaning that IsoP concentrations are likely to be population-dependent. The group concluded that the molecules would make useful biomarkers in sewage to assess human behaviour and health.

Although Santos et al. (2016)[108] were unable to detect IsoPs in wastewater effluent, it is theorised that through including a deconjugation step, as used in the methods of Ryu et al. (2015 and 2016),[106,109] improved detection may be achieved (Table 1-2). Ryu et al. (2015),[106] developed a method to detect 15-F_{2t}-IsoP in wastewater using immunoaffinity extraction with LC-HRMS. β-glucuronidase was included as a pre-treatment which significantly increased the concentrations of IsoPs available for detection in wastewater influent samples (1-20 ng/L) (Figure 1-4). Ryu et al. (2015),[106] also conducted a comprehensive study determining the correlation between oxidative stress and tobacco consumption based on composite influent samples collected from eleven cities within Europe.[109] Their results showed a good correlation between tobacco consumption and IsoP concentrations detected, which similarly to Santos et al. (2015 and 2016),[107, 108] was dependent on urbanisation.

Table 1-2. Concentrations of isoprostanes which have been detected in wastewater in epidemiology pilot studies.

Method	**Lower detection limit**	**Influent** (ng/L)	**Effluent** (ng/L)
ELISA[107, 108]	0.005 ng/mL	1.0-16	ND
IAC-LC-HRMS[106, 109]	MQL 0.3 ng/mL IQL 0.1 ng/mL	18.9-23.3	N/A

MQL = Method quantification limit; IQL = Instrument quantification limit; ND = Not detected; N/A = Not applicable

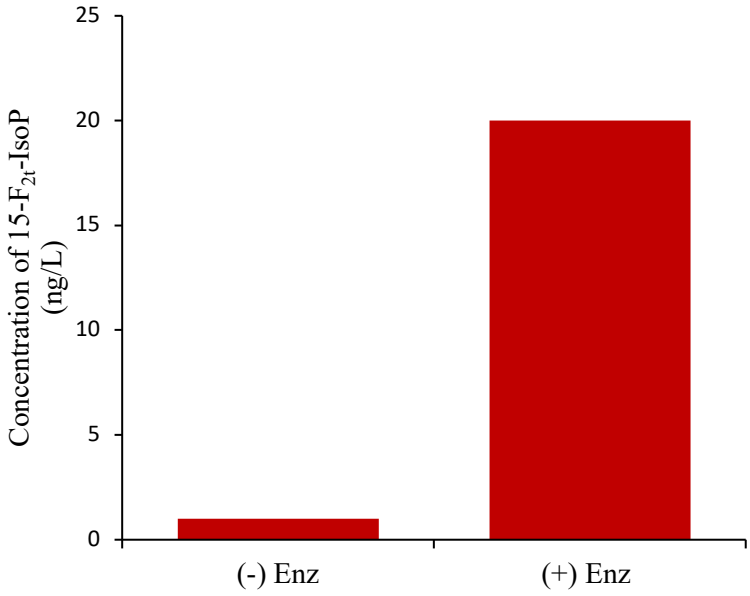


Figure 1-4. The effect of enzymatic deconjugation on 15-F_{2t}-IsoP concentrations in sewage. Sourced from Ryu et al. (2015).[106]

1.5 Isoprostanes as potential emerging contaminants

Emerging contaminants and wastewater epidemiology biomarkers share many of the same characteristics (Section 1.2.2). Although research has begun assessing the concentrations of IsoPs for their use as epidemiology markers,[106, 107, 108] wastewater analysis of IsoPs has not been conducted with consideration to their potential roles as ECs capable of inducing negative biological effects.[18,54] WWTPs represent a portal for the entry of many contaminants to the environment. Based on their biological activity, chemical stability, and presence in wastewater, there is the potential for IsoPs to be ECs of concern.[18,54]

It has already been noted that IsoPs have potent biological activities, and are involved in multiple pathways which have both positive and negative physiological consequences (Section 1.3.4). From a clinical perspective, it has been theorised that external exposure to these compounds through pharmacological or toxicological application to the body has the potential to alter various physiological and pathophysiological processes.[60] As they are already postulated to be involved in the pathogenesis of certain diseased states, there is the further possibility that an environmental presence of these compounds may also pose an ecological threat. It has been proposed that IsoPs may result in "contagious"[18] or "circular"[54] toxicity, whereby exposure to these compounds through effluent release may cause the propagation of oxidative stress in nearby wastewater-receiving ecosystems. They could then stimulate numerous effects in an exposed organism (such as those outlined in Section 1.3.4), potentially resulting in additional oxidative stress and IsoP production, creating this so-called cycle. These effects will likely only be initiated if the organism possesses biomolecules with which IsoPs can interact. However, the role of IsoPs as potential ECs gains credibility when it is considered that many metabolic processes and receptors are phylogenetically conserved between species. For example, aquatic organisms have been found to possess prostanoid molecules and receptors,[110, 111] while IsoPs have also been detected within aquatic organisms in association with lipid peroxidation.[112, 113, 114] Given what is currently understood regarding their bioactivity, IsoPs are likely to exert some of their biological effects via this prostanoid receptor in aquatic species, as they do in others,[54] resulting in a similar plethora of physiological impacts.

To summarise, there are multiple factors which contribute to the emerging status of IsoPs as contaminants of concern, including: (I) They are bioactive compounds capable of inducing adverse effects; (II) Metabolic processes are conserved between species, meaning that it is likely that IsoP-biomolecule interactions will also be conserved in aquatic organisms, potentially allowing these effects to be induced at elevated IsoP concentrations; (III) They are excreted by humans and other animals, whereby they enter WWTPs and have the potential to be discharged into the environment; and (IV) Their continuous release means that there is the potential for pseudo-persistence in environmental matrices. This could result in chronic exposure for aquatic species, by which low levels over a long period of time could result in a continuous cycle of oxidative stress. Further research is required to determine the

concentrations of IsoPs in effluent and whether their presence can result in increases in their plasma concentrations in organisms that are exposed under environmental conditions.[18, 54]

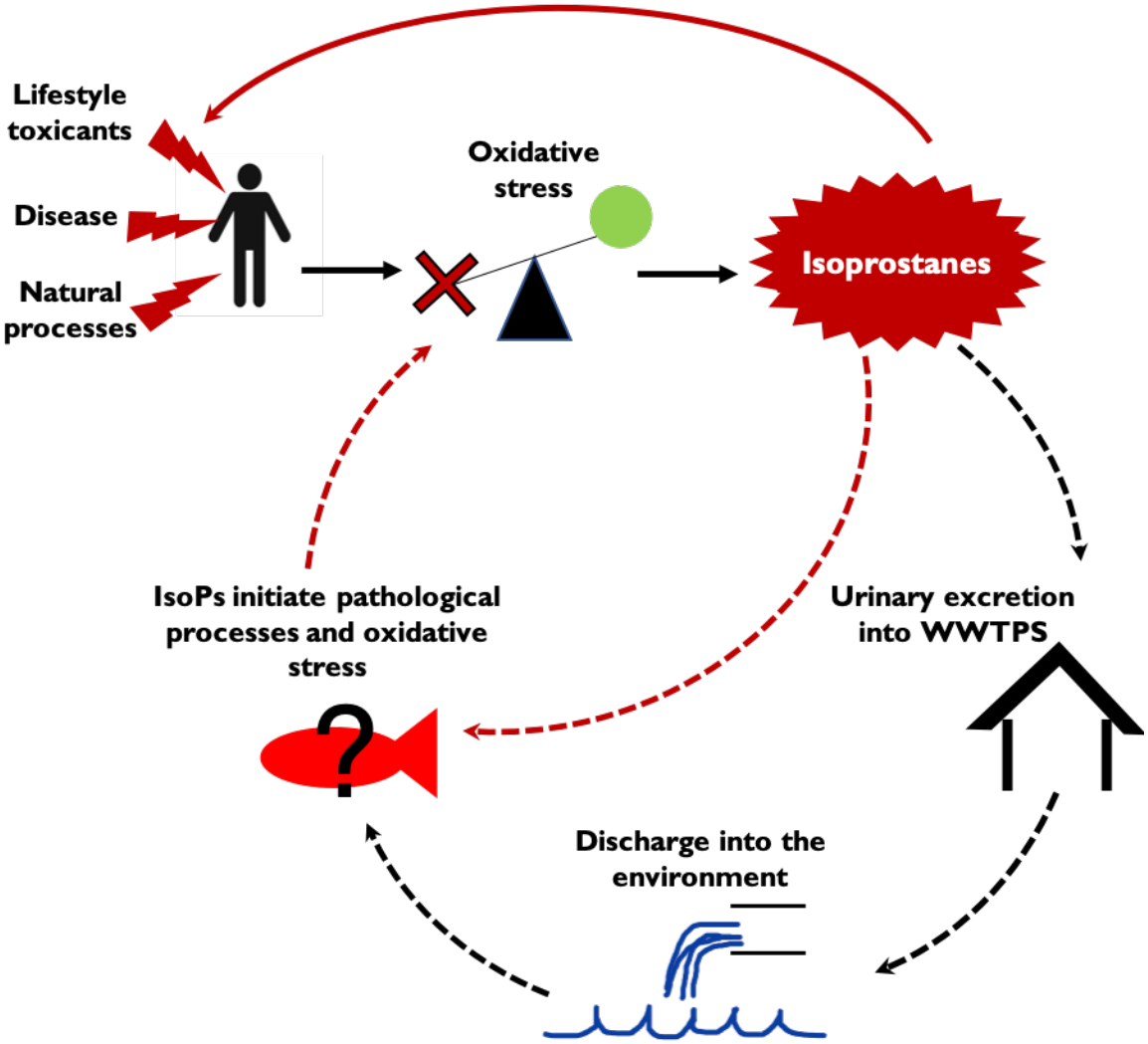


Figure 1-5. A conceptual diagram illustrating the roles of IsoPs as potential emerging contaminants. Solid lines: Represent understood biological pathways of IsoPs. Dashed lines: Represent the hypothesised route of IsoPs as emerging contaminants.

1.6 Thesis objectives and layout

1.6.1 Thesis objectives

Isoprostanes are potential ECs of concern. Bioactive markers of oxidative stress, these compounds have been linked to various biological processes and diseases. The urinary excretion of IsoPs provides a mechanism for their entry to WWTPs, and potentially the wider environment through the discharge of wastewater. This investigation into IsoPs as potential ECs is part of a wider project affiliated with Professor Chris Metcalf at Trent University (Canada), who is currently conducting the first ecotoxicology studies on juvenile trout. This research aims to determine if WWTPs allow for the entry of IsoPs into the environment, through:

1. Developing a method using SPE and GC-MS for the detection of IsoPs in wastewater influent, effluent and freshwater.
2. Measuring the concentration of IsoPs present in wastewater influents and effluents from the Canterbury region.

1.6.2 Layout

Chapter 1 introduces the reader to the aims of this thesis and the research behind the theory of IsoPs as a new potential class of contaminants. In Chapter 2, the development and validation of a GC-MS method for the analysis of 15-F_{2t}-IsoP into wastewater is described. Chapter 3 provides a detailed summary of the sampling plan and the optimised analytical methods used in this study. The provisional results from a survey of Canterbury WWTPs are reviewed in Chapter 4, and finally Chapter 5 discusses the roles of IsoPs as potential ECs, the limitations of this research, and provides recommendations for future work.

Chapter 2

Method Development and Validation

2.1 Chemicals and materials

All solvents used in this study were HPLC grade and purchased from Thermo Fisher Scientific. These included acetone, acetonitrile, hexane, iso-octane and methanol. Ultra-pure water (<18 MQ) was sourced from an in-house Rephile Bioscience Ltd filtration system. The deionised water (DI) was also retrieved from an in-house filtration system. Vircon and Decon-90 were also purchased from Thermo Fisher Scientific.

15-F_{2t}-isoprostane (15-F_{2t}-IsoP) and 15-F_{2t}-isoprostane-D_4 (D_4-15-F_{2t}-IsoP) were purchased from Cayman Chemical and stored at -20°C. All standards were made up in HPLC grade solvents, and purged with nitrogen following use. Step-wise dilutions were used to ensure that accurate concentrations were achieved. BG100® β-glucuronidase (*Helix promatia*) was purchased from Kura Biotec. Pentafluorobenzyl bromide (PFB-Br), diisopropylethylamine (DIPEA), diethylamine (DEA), chlorodimethyl(3,3,3-trifluoropropyl)silane (CDMTFS), ammonium iodide (NH_4I), 2-mercaptoethanol, N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) (≥ 98.5%) were purchased from Sigma Aldrich. The Whatman GG/C filter paper (1.2 um pore size, 47 mm diameter) were also purchased from Sigma Aldrich. Strata X-AW, Strata X and Strata Florisil pesticide grade (FLPR) cartridges (all 500mg/6mL) were purchased from Phenomenex,

Solid phase extraction (SPE) was undertaken on a VacMaster Sample Procession Station (Biotage). The sample bottles were connected to the SPE cartridges through Teflon transfer tubes and end caps. Teledyne-ISCO 3700 portable samplers and PTFE bottles were borrowed from the Institute of Environmental Science and Research (ESR) for wastewater collection. The sampling stainless steel buckets (15 L) and mixer were purchased from Bunnings Warehouse.

2.2 The selection of 15-F_{2t}-Isoprostane as the analyte of interest

For the purpose of this research, the 15-F_{2t}-IsoP isomer was investigated. This isomer was chosen due to its commercial availability and comparability to other literature methods, as it is one of the most commonly analysed IsoP isomers in biological matrices (e.g. urine and plasma).[115, 116] IsoPs serve as proxies for each other,[17] whereby for the purpose of this research, the analysis of 15-F_{2t}-IsoP represents a proxy for the analysis of IsoPs in general and their potential concentrations in wastewater.

2.3 Development of a derivatisation and analytical method for isoprostane analysis

Initially, DIMETRIS, a recently developed derivatisation agent, was trialled to improve the detection of 15-F_{2t}-IsoP through GC-MS analysis (Section 2.3.1). A series of the potential ions that may have formed were calculated while working with this agent, although ultimately none were found. Following which, the more commonly used derivatisation agent BSTFA was trialled alongside PFB-Br. Both BSTFA and MSTFA result in the esterification of a TMS (trimethylsilyl) group on to the analyte. Because there was no record in literature of MSTFA being used for IsoPs and the stability of the agent, it was introduced and trialled as an alternative agent for use alongside the PFB-Br (Section 2.3.2).

2.3.1 Trialling DIMETRIS as a new derivatisation agent for isoprostanes

Synthesis of DIMETRIS

DIMTRIS (Figure 2-1) was synthesised following the methods of Caban et al. (2013).[117, 118] From a bulk solution, 2 mL of acetone:hexane (1:1, v/v) and 300 µl of DEA were pipetted into a glass centrifuge tube and mixed. Then 300 µl of CDMTFS was added, and the mixture vortexed for 5 minutes. Following this, the mixture was centrifuged for 15 minutes at 2500 rpm, as recommended by Caban et al. (2013).[117, 118] Due to only partial phase separation, the methods were adjusted, increasing the centrifuge time to 20 minutes. The pale, yellow supernatant was then transferred into reacti vials, flushed with N_2 and refrigerated (4°C) until use. The agent could be stored for a week before degradation was a concern.

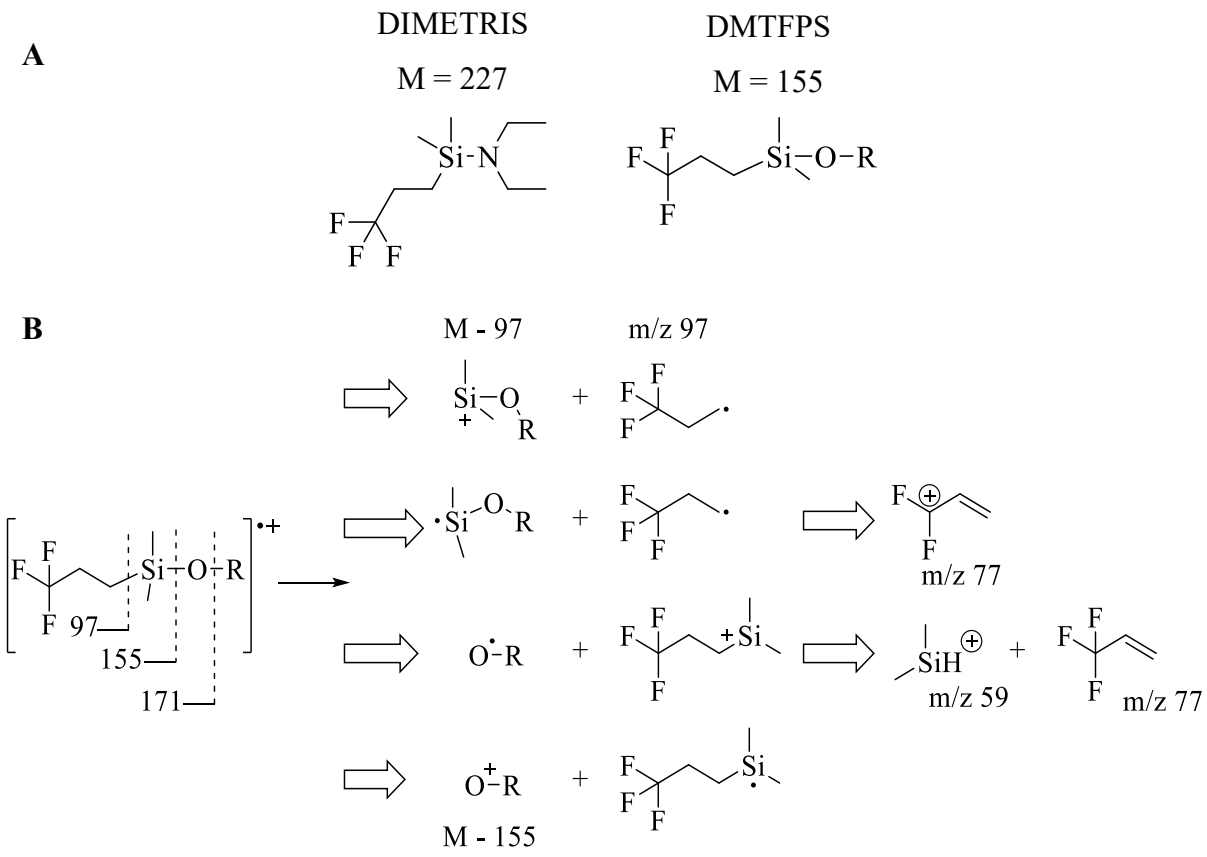


Figure 2-1. (A) The structure of DIMETRIS and DMTFPS, its esterified form. (B) The fragmentation pattern of DMTFPS, resulting in m/z 171, 155 and 97 as the predominant ions. Adapted from Caban et al.(2013).[117, 118]

Derivatisation of 15-F_{2t}-Isoprostane with DIMETRIS

As DIMETRIS was initially developed for the analysis of ethinylestradiol (EE2), derivatisation trials were conducted with both IsoP and EE2, so that the EE2 could be used to determine derivatisation success. First, 500 µl of 1 µg/mL IsoP or EE2 standard were transferred into 1 mL reacti vials, and dried down under N_2. 50 µl of synthesised DIMETRIS and 50 µl of toluene were then added, and the mixture vortexed for 1 minute. The solutions were then derivatised at 30°C for 30 minutes. The derivatised compounds were transferred into GC vials with 400 µl of iso-octane and analysed by the GC-MS.

As no obvious peaks indicative of the IsoP compound were found, a series of the most likely ions formed, were calculated based on: (I) Potential sites of esterification (Figure 2-2); (II) how IsoP molecules fragment in literature (Figure 2-3); (III) how IsoPs fragment with alternative derivatisation agents (Table 2-1); and (IV) the most likely number of esterification's of

DIMETRIS onto the IsoP (Figure 2-4, Table 2-2). In addition to searching for these ions within previous chromatograms, multiple series of these ions were also targeted using selected ionisation mode (SIM) on the GC-MS (summarised in Table 2-3). Other parameters were also adjusted. These included, increasing the incubation time to 45 minutes, trialling higher concentrations of IsoP, increasing the ion source temperature and MS temperature profile. The latter two parameter changes were deemed necessary due to the high predicted mass (660-970) of the esterified parent IsoP compound, which may have affected its volatilisation.[119]

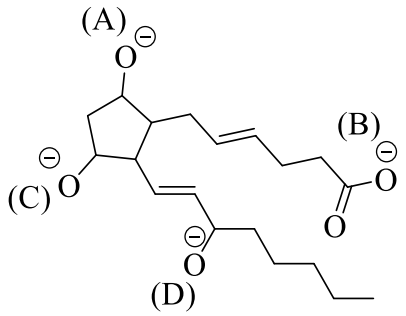


Figure 2-2. Potential sites for esterification of derivatisation agents to 15-F_{2t}-IsoP (A, B, C and D).

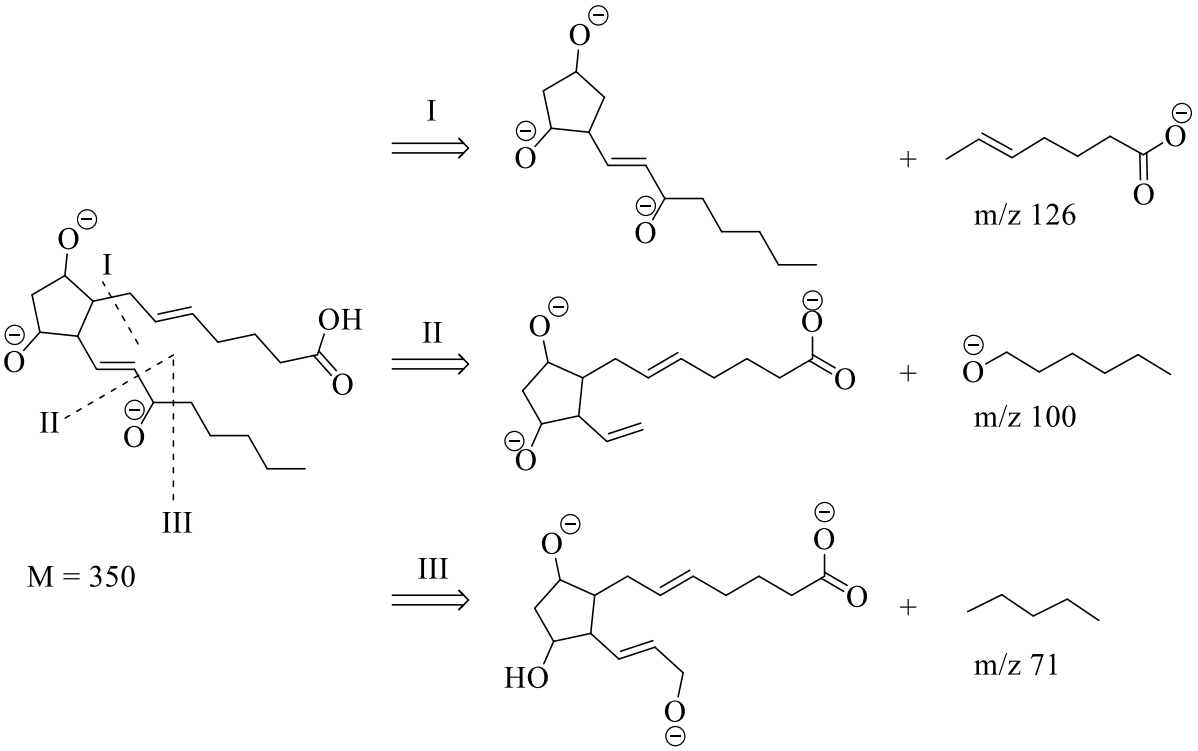


Figure 2-3. Potential fragmentation patterns of 15-F_{2t}-IsoP through GC-MS analysis (Based on Morrow et al. (1990)).[41]

Table 2-1. The mass fragments most likely to be formed based on the behaviour and ionisation patterns observed through the GC-MS analysis of IsoP(TMS)$_3$ (Based on Tsikas et al. (1998)[95]).*

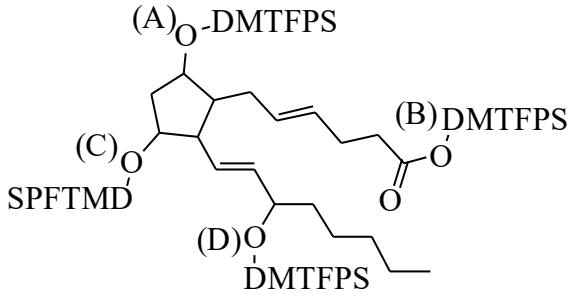
D = DMTFPS	**4 x Deriv**	**3 x Deriv**	**2 x Deriv**	**1 x Deriv**
DOH	171	171	171	171
D	155	155	155	155
P	970	815	660	505
P-DOH-D	644	489	334	179
P-2DOH-D	473	318	-	-
P-3DOH-D	302	-	-	-
P-2DOH-2D	318	-	-	-
P-3DOH-D-CO_2	258	-	-	-
P-2DOH-D-CO_2	429	274	-	-
P-3DOH-CO_2	413	258	-	-
P-2DOH-CO_2	584	429	274	-
P-DOH-CO_2	755	600	445	290

*Based on literature, it is assumed that (A, C, and/or D) are derivatised primarily over (B). i.e. (B) is only derivatised when the IsoP molecule has been derivatised 4 times.

P = Parent compound; D = DMTFPS; DOH = DMTFPS and a hydroxyl group; and Deriv = Number of times derivatised.

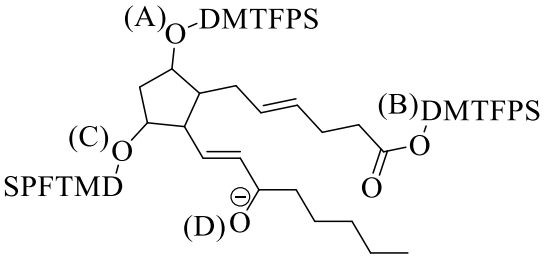
Derivatised 4 times with DMTFPS

(A, B, C, D)

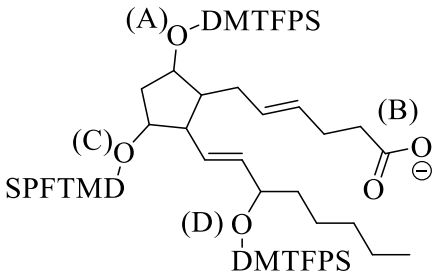


Derivatised 3 times with DMTFPS

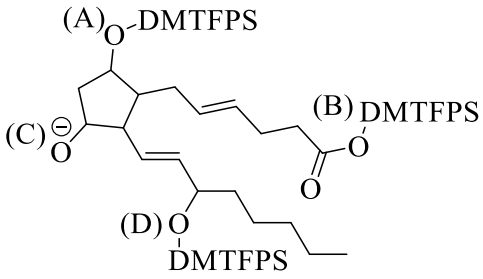
(A, B and C)



(A, C and D)



(A, B and D)



(B, C and D)

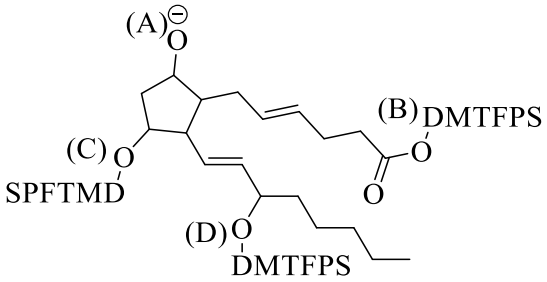
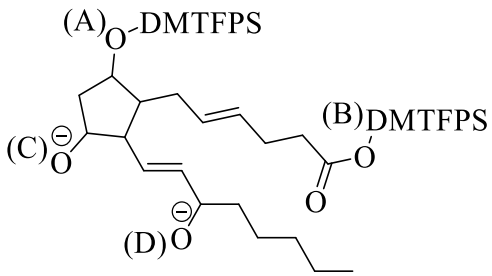


Figure 2-4. The structure of 15-F_{2t}-IsoP derivatised 2-4 times at different sites on molecule.

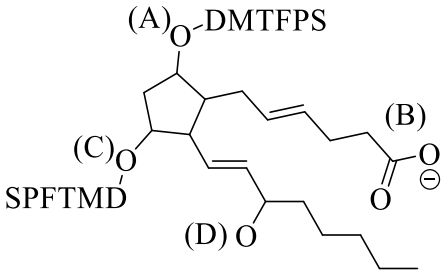
Derivatised 2 times with

DMTFPS

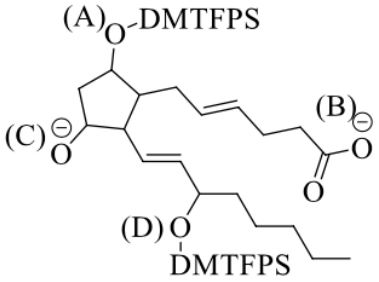
(A and B)



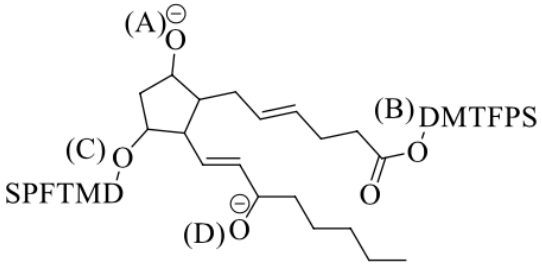
(A and C)



(A and D)



(B and C)



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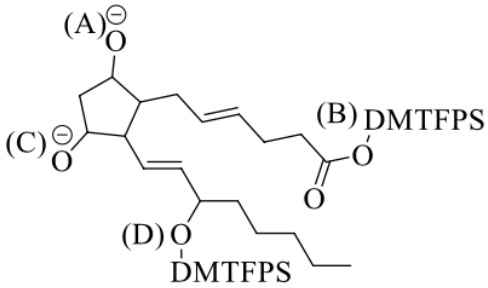

(C and D)

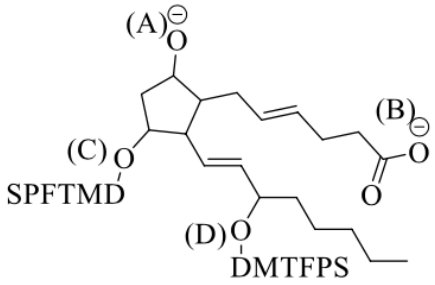

Figure 2-4 continued. The structure of 15-F_{2t}-IsoP derivatised 2-4 times at different sites on molecule.

Table 2-2. A condensed list of the potential ions which may form during GC-MS analysis of 15-F_{2t}-IsoP(DMTFPS)$_n$. This is based on the potential number of esterification's with DMTFPS (altering parent M) and fragment patterns of 15-F_{2t}-IsoP (loss of I, II, and III).

Parent ion	**I**	**II**	**III**
970 = M	(A, B, C, D)	(A, B, C, D)	(A, B, C, D)
	592 = M – 281 – 97	618 = M – 255 – 97	802 = M – 71 – 97
	534 = M – 281 – 155	560 = M – 255 – 155	744 = M – 71 – 155
	518 = M – 281 – 171	544 = M – 255 – 171	728 = M – 71 – 171
	491 = M – 281 – 194	521 = M – 255 – 194	705 = M – 71 – 194
	379 = M – 281 – 310	405 = M – 255 – 310	589 = M – 71 – 310
	347 = M – 281 – 342	373 = M – 255 – 342	557 = M – 71 –342
	398 = M – 281 – 291	424 = M – 255 – 291	608 = M – 71 – 291
	224 = M – 281 – 465	250 = M – 255 – 465	434 = M – 71 – 465
	176 = M – 281 – 513	202 = M – 255 – 513	386 = M – 71 – 513
	301 = M – 281 – 388	327 = M – 255 – 388	511 = M – 71 – 388
	69 = M – 281 – 620	95 = M – 255 – 620	279 = M – 71 – 620
	5 = M – 281 – 684	31 = M – 255 – 684	215 = M – 71 – 684
815 = M			
	(A, B, C), (A, B, D) and (B, C, D)	(A, B, D), (A, C, D) and (B, C, D)	(A, B, C), (A, B, D), (A, C, D) and (B, C, D)
	437 = M = 281 – 97	463 = M – 255 – 97	647 = M – 71 – 97
	379 = M = 281 – 155	405 = M – 255 – 155	589 = M – 71 – 155
	363 = M – 281 – 171	389 = M – 255 – 171	573 = M – 71 – 171
	340 = M – 281 – 194	366 = M – 255 – 194	550 = M – 71 – 194
	224 = M – 281 – 310	250 = M – 255 – 310	434 = M – 71 – 310
	192 = M – 281 – 342	218 = M – 255 – 342	402 = M – 71 – 342
	243 = M – 281 – 291	269 = M – 255 – 291	453 = M – 71 – 291
	69 = M – 281 – 465	95 = M – 255 – 465	279 = M – 71 – 465
	21 = M – 281 – 513	47 = M – 255 – 513	231 = M – 71 – 513
	(A, C, D)	(A, B, C)	-
	592 = M – 126 – 97	618 = M – 100 – 97	-
	534 = M – 126 – 155	560 = M – 100 – 155	-
	518 = M – 126 – 171	544 = M – 100 – 171	-
	495 = M – 126 – 194	521 = M – 100 – 194	-
	379 = M – 126 – 310	405 = M – 100 – 310	-
	347 = M – 126 – 342	373 = M – 100 – 342	-
	398 = M – 126 – 291	424 = M – 100 – 291	-
	224 = M – 126 – 465	250 = M – 100 – 465	-
	176 = M – 126 – 513	202 = M – 100 – 513	-

660 = M			
	(A, B), (B, C) and (B, D)	(A, B), (B, C)	(A, B), (B, C), (A, D), (C, D) and (B, D)
	282 = M – 281 – 97	463 = M – 100 – 97	492 = M – 71 – 97
	224 = M – 281 – 155	405 = M – 100 – 155	434 = M – 71 – 155
	208 = M – 281 – 171	389 = M – 100 – 171	418 = M – 71 – 171
	185 = M – 281 – 194	366 = M – 100 – 194	395 = M – 71 – 194
	69 = M – 281 – 310	250 = M – 100 – 310	279 = M – 71 – 310
	37 = M – 281 – 342	218 = M – 100 – 342	247 = M – 71 – 342
	(A, C), (A, D) and (C, D)	(A, D), (C, D) and (B, D)	-
	437 = M – 126 – 97	308 = M – 255 – 97	-
	379 = M – 126 – 155	250 = M – 255 – 155	-
	363 = M- 126 – 171	234 = M – 255 – 171	-
	340 = M – 126 – 194	211 = M – 255 – 194	-
	224 = M – 126 – 310	95 = M – 255 – 310	-
	192 = M – 126 – 342	63 = M – 255 – 342	-
	176 = M – 126 – 513	202 = M – 100 – 513	-
	(A, B), (B, C) and (B, D)	(A, B), (B, C)	(A, B), (B, C), (A, D), (C, D) and (B, D)
	282 = M – 281 – 97	463 = M – 100 – 97	492 = M – 71 – 97
	224 = M – 281 – 155	405 = M – 100 – 155	434 = M – 71 – 155
	208 = M – 281 – 171	389 = M – 100 – 171	418 = M – 71 – 171
	185 = M – 281 – 194	366 = M – 100 – 194	395 = M – 71 – 194
	69 = M – 281 – 310	250 = M – 100 – 310	279 = M – 71 – 310
	37 = M – 281 – 342	218 = M – 100 – 342	247 = M – 71 – 342
	(A, C), (A, D) and (C, D)	(A, D), (C, D) and (B, D)	-
	437 = M – 126 – 97	308 = M – 255 – 97	-
	379 = M – 126 – 155	250 = M – 255 – 155	-
	363 = M- 126 – 171	234 = M – 255 – 171	-
	340 = M – 126 – 194	211 = M – 255 – 194	-
	224 = M – 126 – 310	95 = M – 255 – 310	-
	192 = M – 126 – 342	63 = M – 255 – 342	-

GC-MS method parameters and analysis

The basic GC-MS instrumental parameters were set according to those described at the beginning of Section 3.2.3.3. Additional parameters were trialled and altered over time to enable the detection of the ions (Table 2-3). Following analysis in SCAN mode, all of the calculated potential ions were searched for within the final chromatograms. Additionally, blank versus standard (1 µg/mL 15-F_{2t}-isoP) chromatograms were compared to see if an obvious peak representative of 15-F_{2t}-IsoP could be identified manually. Ions representative of the EE2 compound were found, but despite this, 15-F_{2t}-IsoP could not be identified with this derivatisation agent. To ensure that the lack of identification was not due to a fault with the IsoP standard, the standards were also run on a mass spectrometer to confirm their identity. Ultimately, due to time restrictions an alternative derivatisation method was trialled (Section 2.3.2).

Table 2-3. GC-MS instrumental parameters for different methods trialled to enable the detection of the 15-F_{2t}-IsoP derivatised with DMTFPS.

	Column Temperature							
Method	**Rate**	**Final**	**Hold time**	**Injection**	**Ion source**	**Interface**	**Acq mode**	**Ions m/z**
D-1	- 10.00	100°C 300°C	5.00 5.00	250°C	200 °C	250°C	Scan	50-700
D-2	- 10.00	150°C 320°C	5.00 10.0	300°C	200°C	300°C	Scan	50-1000
D-3	- 10.00	150°C 320°C	5.00 15.0	300°C	200°C	300°C	Scan	50-1000
D-4	- 10.00	200°C 300°C	5.00 30.0	300°C	200°C	300°C	SIM	59, 97, 255, 299, 316, 444, 445, 471, 488, 489, 643, 815

2.3.2 Trialling PFB-Br and BSTFA or MSTFA as derivatisation agents

2.3.2.1 Derivatisation with PFB-Br and BSTFA or MSTFA

Following the derivatisation trials with DIMETRIS, PFB-Br and BSTFA were then investigated as they are commonly used in literature for the identification of IsoPs. Methods for PFB-Br and BSTFA derivatisation were based on those prescribed in literature.[94, 102, 120] All of the reagents were anhydrous and purged with N_2 following each use then stored in desiccators to reduce moisture contamination. Small volumes of the solvents were sourced from the in-house dry solvent system each day before use. The iso-octane was dried through treatment with solvent washed (3 x rinsed with each of methanol, acetonitrile and acetone) anhydrous sodium sulphate each day and stored in a desiccator.

Initially, 500 µL aliquots of a 1 µg/mL 15-F_{2t}-IsoP standard were transferred into reacti vials and dried down under N_2. 100 µL of anhydrous acetonitrile was then added to the samples and derivatisation agent blanks. These were then mixed before 10 µL of methanol followed by 10 µL of PFB-Br and DIPEA were also added. The vials were then incubated at 30°C for 60 minutes. Following cooling, the mixtures were evaporated under N_2. 30 µL of BSTFA, BSFTA + TMS (99:1) or MSTFA (see Section 3.2.3.1 for synthesis) were added to the vials, and they were incubated for an additional 60 minutes at 60°C. Once cooled to room temperature, the samples were quantitatively transferred into amber glass GC vials with 2 x 250 µL anhydrous iso-octane and stored at 4°C.

GC-MS methods and analysis

Based on the literature, m/z 569 was set as the desired quantifier ion to target 15-F_{2t}-IsoP (Figure 2-5). Both SCAN and SIM modes were used to identify the ion, however no peaks indicative of the ion were observed. New PFB-Br and catalyst were also trialled, as it was concluded that this esterification processes was most likely the stage of the reaction that was not working as expected, but this still did not result in finding the ion. Multiple different instrumental parameters were also trialled in order to accurately identify the compound (Table 2-4). These included altering the temperature profile and searching for alternative ions that appeared in the SCAN chromatograms of the samples versus blanks (Table 2-4, Method P-2a). Reactions with or without the presence of PFB-Br or the catalyst were also conducted. Through these trials, a different peak and series of ions were identified that were linked to the formation of 15-F_{2t}-IsoP-$(TMS)_4$, leading to the conclusion that the initial PFB esterification reaction was

not occurring. The 15-F_{2t}-IsoP(TMS)$_4$ formation was confirmed through a similarity search of a standard and derivatisation agent blank, identifying m/z 391, 462, 481, 537, 552, 571, 627 as likely being representative of the compound. Further method development on the basis of these findings was then initiated (Section 2.3.2.2).

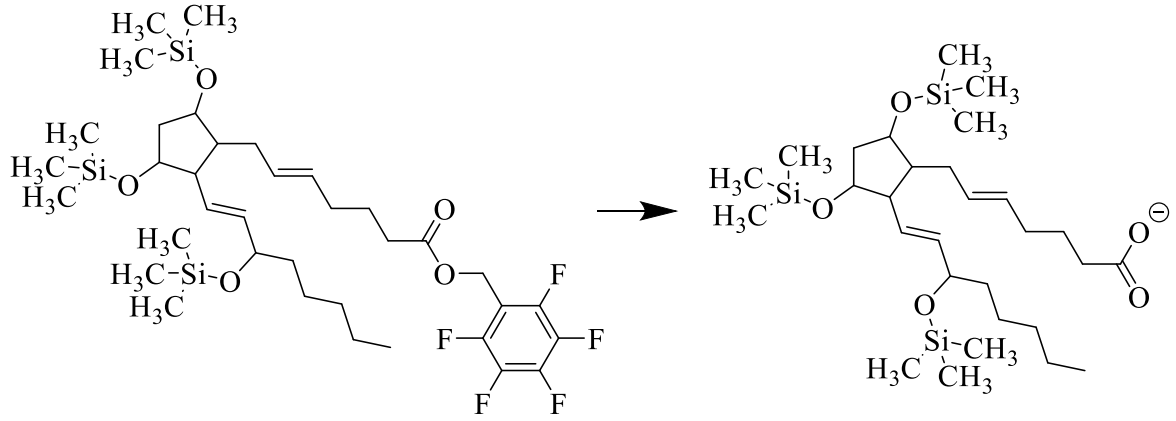

Figure 2-5. Ionisation reaction of IsoP(TMS)(PFB) to form 15-F_{2t}-IsoP(TMS)$_3$ (m/z/ 569) through GC-MS analysis.

Table 2-4. GC-MS instrumental parameters for the different methods trialled to enable the detection of the isoprostanes derivatised with PFB-Br and BSTFA or MSTFA.

	Column Temperature							
Method	**Rate**	**Final**	**Hold time**	**Injection**	**Ion source**	**Interface**	**Acq mode**	**Ions m/z**
P-1[95]	- 25 2	80°C 250°C 280°C	2.00 0.00 5.00	280°C	140°C	280°C	Scan	50-1000
P-2a[121]	- 10.00 20.00	150°C 190°C 310°C	4.00 0.00 15.00	280°C	150°C	310°C	Scan	50-1000
P-2b	- 10.00 20.00	150°C 190°C 310°C	4.00 0.00 15.00	280°C	150°C	310°C	SIM	569
P-2c	- 10.00 20.00	150°C 190°C 310°C	4.00 0.00 15.00	280°C	150°C	310°C	SIM	147, 173, 191, 237, 257, 391, 462

2.3.2.2 *Optimising the derivatisation reaction with either MSTFA or BSTFA to form 15-F_{2t}-IsoP(TMS)$_4$*

Both MSTFA and BSTFA were trialled as derivatisation agents to produce 15-F_{2t}-IsoP(TMS)$_4$. MSFTA was selected for further method development due to the reliable stability of the samples following derivatisation. Their relative stabilities were determined by analysing standards of 1 µg/mL and 0.1 µg/mL 15-F_{2t}-IsoP derivatised with either BSTFA or MSTFA daily for a week and comparing the changes in signal intensity. While the general working concentration of 15-F_{2t}-IsoP was 1 µg/mL to ensure optimisation of the derivatisation methods, a 10 point calibration curve was also formed with different concentrations of 15-F_{2t}-IsoP (1-1000 ng/mL) to ensure linearity. To derivatise the compounds, aliquots of the IsoP standards were dried down in reacti vials and 30 µL of the MSTFA mix was added. Following mixing, the standards were incubated for 45 minutes at 65°C. Once cooled, the samples were then transferred into 200 µL GC vial inserts, and made up to either 100 µL or 200 µL with iso-octane and analysed by GC-MS. Ultimately the final volume of 200 µL was selected to improve the transfer of the derivatised analytes into the GC vials.

GC-MS methods and analysis

Multiple instrumental parameters (Table 2-5) were trialled before method M-1c was selected as an optimised method which could then be carried through to other stages of the method development (extraction, deconjugation, quantifying internal standard and surrogate). While other ions had been identified as being representative of 15-F_{2t}-IsoP, they were also formed as a result of the derivatisation agent and were not used as qualifier ions in case they resulted in the false detection of the analyte. The initial oven temperature was held at 140°C for 4 minutes, then increased at a rate of 18°C/minute to 300°C. It was held for an additional 4 minutes, then increased to 310°C, at a rate of 20°C/minute. The samples were injected at a temperature of 250°C, the ion source held at 200°C and the interface at 310°C. The m/z 391 was used as the quantifier ion, while 481 and 537 were used as the qualifiers (Figure 2-6). The retention time of the 15-F_{2t}-IsoP(TMS)$_4$ ions were 13.62 minutes, although the GC-MS instrumental parameters were later adjusted to allow for a longer temperature profile in order to separate interferences caused by the deconjugation agent which occurred at the retention time of the analyte (Section 2.2.2).

Table 2-5. GC-MS instrumental parameters for different methods trialled to enable the detection of the isoprostanes derivatised with BSTFA or MSTFA.

	Column Temperature							
Method	**Rate**	**Final**	**Hold time**	**Injection**	**Ion source**	**Interface**	**Acq mode**	**Ions m/z**
P-2a[121]	- 10.00 20.00	150°C 190°C 310°C	4.00 0.00 15.00	280°C	150°C	310°C	Scan	50-1000
P-2b	- 10.00 20.00	150°C 190°C 310°C	4.00 0.00 15.00	280°C	150°C	310°C	SIM	391, 462, 481, 537, 552, 571, 627
P-2c	- 10.00 20.00	150°C 190°C 310°C	4.00 0.00 15.00	280°C	150°C	310°C	SIM	391, 481, 552, 571, 627
M-1a[76]	- 18.00 20.00	140°C 300°C 310°C	4.00 4.00 5.00	250°C	200°C	310°C	SIM	391, 462, 481, 537, 552, 571, 627
M-1b	- 18.00 20.00	140°C 300°C 310°C	4.00 4.00 5.00	250°C	200°C	310°C	SIM	391, 481, 552, 571, 627
M-1c	- 18.00 20.00	140°C 300°C 310°C	4.00 4.00 5.00	250°C	200°C	310°C	SIM	***391***,* 481, 537

*Quantifier ion italicised in bold

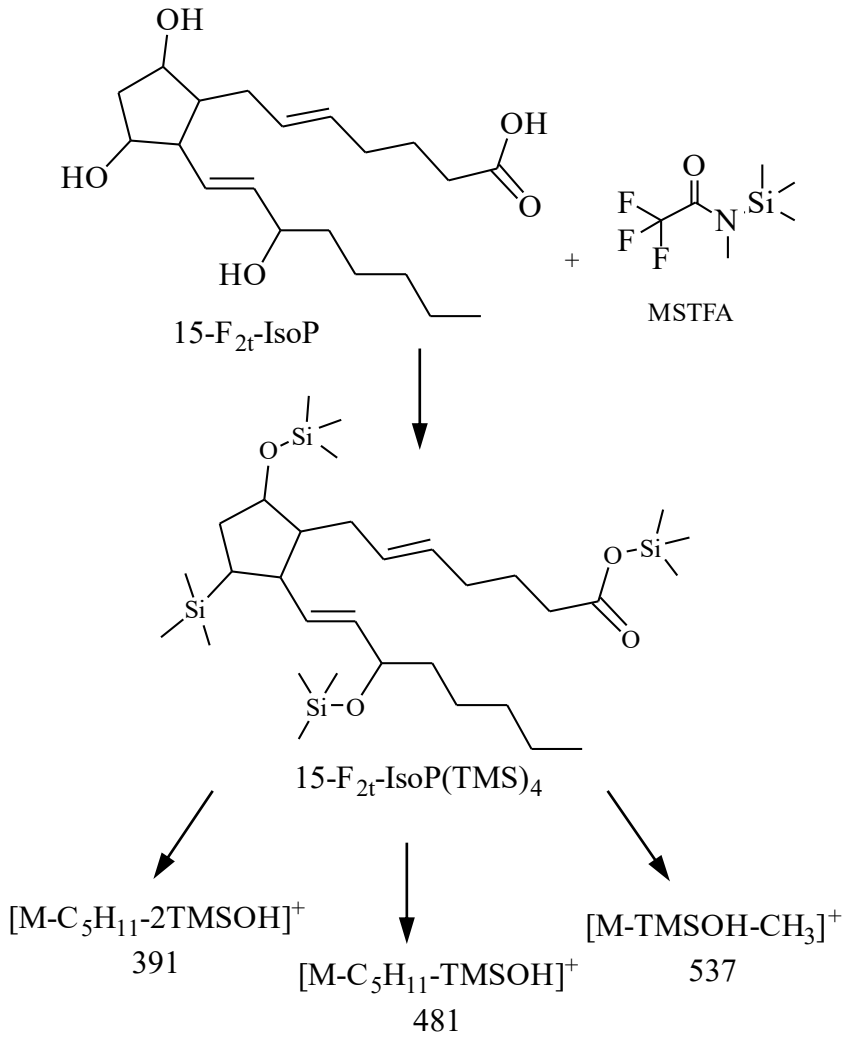

Figure 2-6. Derivatisation of 15-F_{2t}-IsoP with MSTFA, and the subsequent formation of the quantifier (m/z 391) and qualifier (m/z 481 and 537) ions used for identification.

2.4 Filtration, deconjugation and solid phase extraction development

2.4.1 Filtration

Initially, due to the small volume (200 mL) of wastewater being used, extraction was trialled without vacuum filtration. But due to the clogging of the SPE cartridges, filtration was introduced. For each sample, a 500 mL aliquot of wastewater effluent was defrosted then adjusted to pH 5 with acetic acid to ensure they were at the optimum pH for the deconjugation reaction (Section 2.4.2). The samples were vacuum filtered through solvent cleaned GF/C 47 mm filter papers. When working with influent, only 100 mL was measured and filtered per filter paper to ensure efficiency. In comparison, 200 mL of effluent could be filtered per filter paper due to the presence of less organic matter. The bottle and filter funnel were then rinsed

with an additional 20 mL ultrapure water and allowed to flow through. The filter papers were then dried and stored on methanol rinsed tinfoil in the freezer. Following filtration, the pH was checked again, and if required extra acetic acid was added.

2.4.2 β-glucuronidase deconjugation method development

Method Development

As a large proportion of 15-F_{2t}-IsoP molecules are excreted as glucuronide conjugates, deconjugation treatment can significantly increase the concentrations available for detection (Section 1.4.1).[105, 106] Deconjugation was conducted following the filtration of the wastewater samples. The deconjugation trials were run alongside each extraction trial (Section 2.4.3), starting with Trial 1 (Section 2.4.3.1). To ensure comparability, every trial was set up as outlined in Table 2-6, and the respective sample codes are applied throughout the rest of this chapter. The wastewater was split into 4 x 200 mL aliquots for each SPE cartridge type, while an additional 2 x 200 mL DI was also prepared. The glucuronidase enzyme was then assessed in: (I) Spiked wastewater (SW-En); (II) unspiked wastewater (W-En); (III) Spiked DI (SDI-En); and (IV) unspiked DI (DI-En) (Table 2-6). Two additional aliquots of 200 mL wastewater were also extracted without the addition of the enzyme to determine the degree of interference from the enzyme during the extraction procedure and analysis (SW and W). Once the wastewater had been separated into aliquots, 1.5 mL of β-glucuronidase was added to the appropriate samples (Table 2-6). These were then placed in a water bath and incubated at 37°C for 2 hours.[105, 106, 122] Once cooled, these samples were spiked with the surrogate and run through the extraction trials (Section 2.4.3).

GC-MS analysis and results

GC-MS analysis was conducted following the method M1c, above (Section 2.3.2.2, Table 2-5). The presence of β-glucuronidase resulted in a peak at a very similar retention time to the analyte, so to separate their retention times, the temperature profile was adjusted from a rate of 18°C/minute from 140°C to 300°C, to 16°C/minute. As a result, the IsoP retention time increased to 14.5 minutes and could be better quantified in the presence of the deconjugation agent. However, this temperature profile was subsequently adjusted again to attempt to separate some of the matrix effects in the following trials (Section 2.4.3.1).

Table 2-6. A representation of the minimum number of sample-types run for each extraction trial. This allowed the effects of the matrix on the recoveries and reproducibility to be determined and compared within and between trials.

Sample code*	**Sample Type** (200 mL)	**Enzyme** (1.5 mL)	**Native** 15-F_{2t}-IsoP (200 µl 0.1 µg/mL)	**Surrogate** D_4-15-F_{2t}-IsoP (200 µl 0.1 µg/mL)
SW-En	Wastewater	✓	✓	✓
W-En	Wastewater	✓	×	✓
SW	Wastewater	×	✓	✓
W	Wastewater	×	×	✓
SDI-En	Deionised water	✓	✓	✓
DI-En	Deionised water	✓	×	✓

*DI = Deionised water; En = Enzyme; S = Spiked; and W = Wastewater

2.4.3 Extraction method development

2.4.3.1 Trial 1: A comparison between SPE cartridges

Trial 1: Methods

The first SPE extraction trial consisted of a comparison between the recoveries of Strata X and Strata X-AW cartridges. Although both have been used in the literature, the former was selected as it is commonly used within our research laboratory and in the literature for the efficient extraction of polar analytes in environmental samples. The latter was selected as it commonly provides good clean-up efficiency and recoveries for IsoPs in biological samples.[100] As noted previously (Table 2-6), the wastewater and DI were split into 200 mL aliquots for the extraction trials. Following the deconjugation treatment, 200 µL of the D_4-15-F_{2t}-IsoP (0.1 µg/mL) surrogate was added to every sample. Then 200 µL of 15-F_{2t}-IsoP (0.1 µg/mL) native was added to 3 aliquots for each cartridge type, representative of: (I) DI spike; (II) wastewater + enzyme spike; and (III) wastewater spike. A comparative for recovery analysis was also dispensed with 200 µL of the 0.1 µg/mL surrogate-native standard mix in 10 mL acetone.

Each cartridge (6 x Strata X and 6 x X-AW) was conditioned prior to extraction. First, 3 x 5 mL acetone was allowed to flow through under gravity. Once half the solvent was through, the flow was stopped for 2 minutes, then continued. This process was repeated with methanol and

ultrapure water. Finally, ultrapure water was added a fourth time, and left to sit covered with methanol-rinsed tinfoil until the samples were loaded. The samples were then loaded and passed through either the Strata X or X-AW cartridges, before being dried under a vacuum for 2 hours. Prior to elution, the cartridges were stacked above previously-used Strata FLPR cartridges filled with solvent-rinsed anhydrous sodium sulfate. Next, 5 mL of acetone was added to the top Strata X or X-AW cartridges and allowed to drip halfway through, then sit for 2 minutes to saturate the SPE bed and eluted. This was repeated an additional 5 times, bringing the total eluent volume to 30 mL. The samples were dried down to ~0.5 mL, and quantitatively transferred into reacti vials, with 2 x 250 µL acetone. These and the comparative standard were then dried down under a gentle stream of N_2 at 40°C for derivatisation with MSTFA (Section 3.3.3 for finalised method).

Trial 1: GC-MS analysis and results

The samples were analysed using the GC-MS instrumental parameters specified in Section 2.4.2. To account for the matrix interferences, the temperature profile was adjusted again to a slower rate of increase. The GC-MS temperature profile was initially held for 4 minutes at 140°C, then increased at a rate of 8°C/minute to 300°C. It was then increased to 310°C at 20°C/minute, and held for an additional 5 minutes. The retention time of the analytes became 21.795 and 21.792 minutes for 15-F_{2t}-IsoP and D_4-15-F_{2t}-IsoP respectively. While this change did provide some improvement, the recoveries were still too low with very poor reproducibility, meaning the extraction procedures required more adjustment before further clean-up steps could be trialled.

Initially, the cartridges were dried for 2 hours once the samples had been loaded. To ensure that moisture contamination was not contributing to the recovery inconsistencies, the drying time was increased to 2.5 and 3 hours which still did not improve the recoveries. The DI-enzyme blank (DI-En) also indicated that the enzyme caused a low level of interference at the retention time of the analytes (Figure 2-7, DI-En). The recovery and reproducibility values were variable for both the DI and the wastewater samples, the wastewater especially resulting in significant matrix effects. The recoveries ranged from 0-60%, with the lower values being the more dominant. Additionally, many peaks were unable to be accurately integrated due to the presence of interfering peaks, inaccurate ion ratios and low signal to noise ratios (S/N < 2.5) (Figure 2-7).

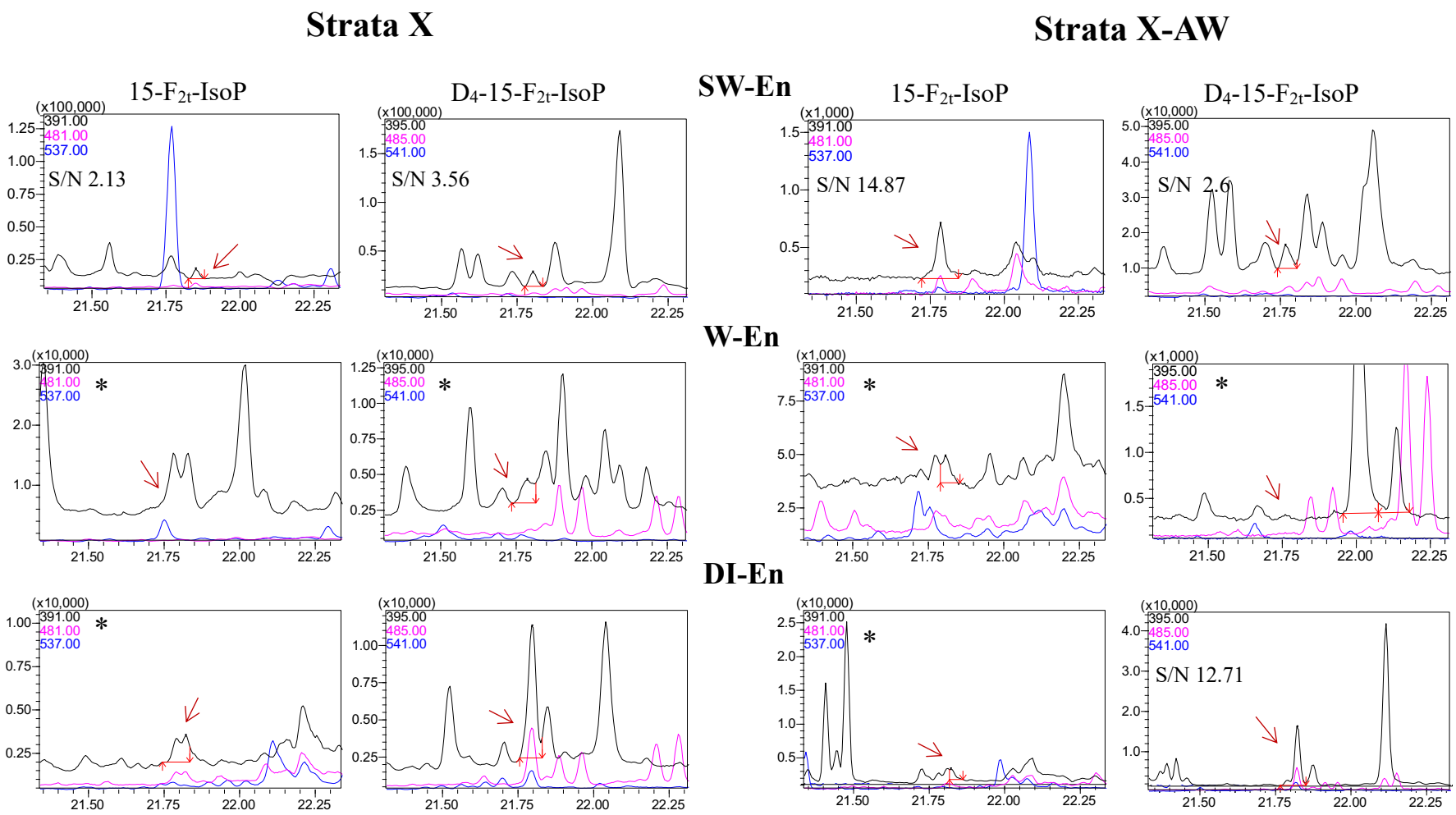

*Figure 2-7. Chromatogram retention windows representative of 15-F_{2t}-IsoP and D_4-15-F_{2t}-IsoP, under different conditions and extracted through either Strata X or X-AW cartridges. Shown above are the results for the extraction of SW-En, W-En and DI-En. *Denotes results where the native or surrogate was not detected due to inaccurate ion ratios, low signal-to-noise ratios or interfering peaks. An arrow is used to depict the location of the IsoP peak. SW-En = Spiked wastewater with enzyme; W-En = Wastewater with enzyme; and DI-En = Deionised water with enzyme.*

2.4.3.2 Trial 2: Refining the extraction method

Trial 2: Methods

The next trial focused on the Strata X-AW cartridges with the addition of an acidic pre-conditioning step, alongside a comparison between methanol or acetone elution, based on the methods of Taylor et al. (2008).[103] First the samples were filtered and deconjugated as described above (Table 2-6). The cartridges were then pre-conditioned with 3 x 5 mL acetone. Following this, 3 x 5 mL of methanol:acetic acid (98:2, v/v) was run through, followed by 5 mL ultrapure water. Each solvent was allowed to flow through under gravity, until half was gone, before the flow was stopped again to allow the solvents to permeate the SPE bed. Following the ultrapure water, an additional 5 mL of water was added to prevent the SPE bed from becoming exposed to air. The samples were then loaded and allowed to dry for 2.5 hours. Following this they were stacked above the sodium sulfate FPLR cartridges and eluted with either 6 x 5 mL methanol or acetone, as described above. Two comparatives were also made up prior to extraction with 200 µL of a 0.1 µg/mL surrogate-native standard mix in either 10 mL acetone or methanol. The samples and comparatives were then dried down at 40°C, and derivatised with MSTFA.

Trial 2: GC-MS analysis and results

The GC-MS parameters developed in Trial 1 (Section 2.4.3.1) were used for the remaining trials. Elution with methanol was selected for further method development as it was found to provide better recoveries and more reproducible results in comparison to acetone (Table 2-7, Figure 2-8). The enzyme, as well as other interfering substances, continued to result in low levels of interference that could not be removed through adjusting the temperature profiles to try to separate their retention times from those of the IsoPs. Matrix effects were also prominent issues in the wastewater samples and impacted the reliability of peak integration by altering the ion ratios. While many of the interferences were variable between samples and treatment plants, Bromley wastewater samples consistently had a trialling peak, with a m/z of 395, which interfered with the detection of the surrogate. A similarity search in SCAN did not yield any suggestions as to what the interfering substance could have been, although some of the interfering substances which were identified included eicosanoic acid, pentacosanoic acid glycerol and androsterone. As a result it was deemed necessary that further sample clean-up would be required to remove some of these compounds and other unknown interferences.

Table 2-7. A comparison between the extraction efficiencies of 15-F_{2t}-IsoP and D_4-15-F_{2t}-IsoP spiked into either wastewater or DI and eluted with either methanol (MeOH) or acetone (Ace).

Data	Sample	SW-En-MeOH	SW-En-Ace	W-En-MeOH	W-En-Ace*	SDI-MeOH	SDI-Ace	DI-En-MeOH	DI-En-Ace
15-F_{2t}-IsoP	Avg.R (%)	83.2	0.3	8.5	ND	39.9	2.5	6.3	ND
	SD	66.7	0.4	14.8	-	33.3	3.6	1.2	ND
D_4-15-F_{2t}-IsoP	Avg.R (%)	81.1	1.3	89.6	76.6	44.1	ND	74.2	14.2
	SD	50.5	1.9	17.1	-	38.4	ND	45.4	20.1

*Only have data for one series of trials. All others were repeated 2-3 times.
ND = Not detected; DI = Deionised water; En = Enzyme; S = Spiked; W = Wastewater; MeOH = methanol; and Ace = acetone.

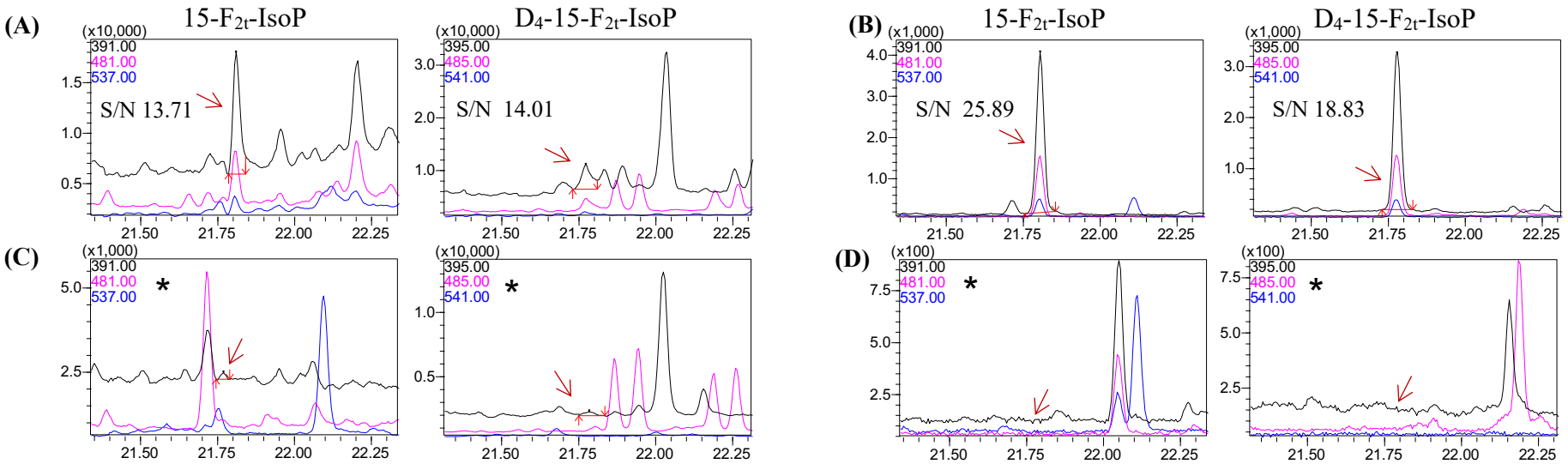

Figure 2-8. Chromatogram comparisons for native (15-F_{2t}-IsoP) and surrogate (D_4-15-F_{2t}-IsoP) spikes in wastewater and DI, using either methanol or acetone to elute the compounds. Of which, methanol elution provided better analyte recoveries. ***(A)*** *SW-En eluted with methanol;* ***(B)*** *SDI-En eluted with methanol* ***(C)*** *SW-En eluted with acetone; and* ***(D)*** *SDI-En eluted with acetone.*Represents no peak or incorrect ion ratios.*

SW-En = Spiked wastewater with enzyme; W-En = Wastewater with enzyme; and DI-En = Deionised water with enzyme.

2.4.3.3 Trial 3: Sample purification

The next series of method development trials focused on introducing a clean-up strategy following sample extraction. Both florisil cartridges and silica gel chromatography were trialled.

Florisil methods and results

For the florisil trial, sample preparation (filtration, deconjugation, and loading of samples onto Strata X-AW cartridges) was conducted following the aforementioned methods (e.g. Section 2.4.3.2). The elution differed from previous methods as the sodium sulfate was placed into new, unused FL-PR cartridges, before it was pre-conditioned with 3 x 5 mL acetone. The samples were then eluted with methanol as in Trial 2, dried down, derivatised and analysed. The results suggested that although florisil removed many of the interfering substances and other contamination within the samples, it also resulted in significant loss of analyte as no native or surrogate 15-F_{2t}-IsoP could be detected through GC-MS analysis (Figure 2-9).

Silica gel chromatography methods and results

The first investigation using silica gel chromatography focused on determining the recoveries of the native and surrogate IsoPs using methods adapted from Milatovic et al. (2011).[123] First, a small volume of glass wool was packed into the glass column. Around 0.55 g of silica gel was then loaded onto the wool and 5 x 1 mL ethyl acetate was used to condition the silica gel, while a pipette bulb was used to expunge the remaining solvent. A spike (200 µl of a 0.1 µg/mL native and surrogate mix) and blank in 1 mL methanol were then prepared and loaded onto two separate silica columns. They were allowed to drip through then the remaining moisture was removed through forcing it out with a pipette bulb. Because Milatovic et al. (2011)[123] used a wash step prior to elution, this was also trialled. Following sample loading, 5 x 1 mL of the ethyl acetate wash was loaded onto the column. The wash was collected in a 5 mL reacti vial for analysis as it was suspected that the ethyl acetate would have resulted in the elution of the analytes due to its polarity. Following this, 5 x 1 mL ethyl acetate:methanol (50:50 v/v) was used to elute the compounds and was collected in 5 mL reacti vials. The extracts were then dried down under nitrogen, derivatised and analysed. A 70% recovery of both the native and surrogate in the wash solvent, compared to <1% in the elution solution suggested that the compounds were being eluted during the wash. It was concluded that the wash would not be included in the methods, and instead once the samples had been loaded, they would be eluted with the ethyl acetate:methanol mix.

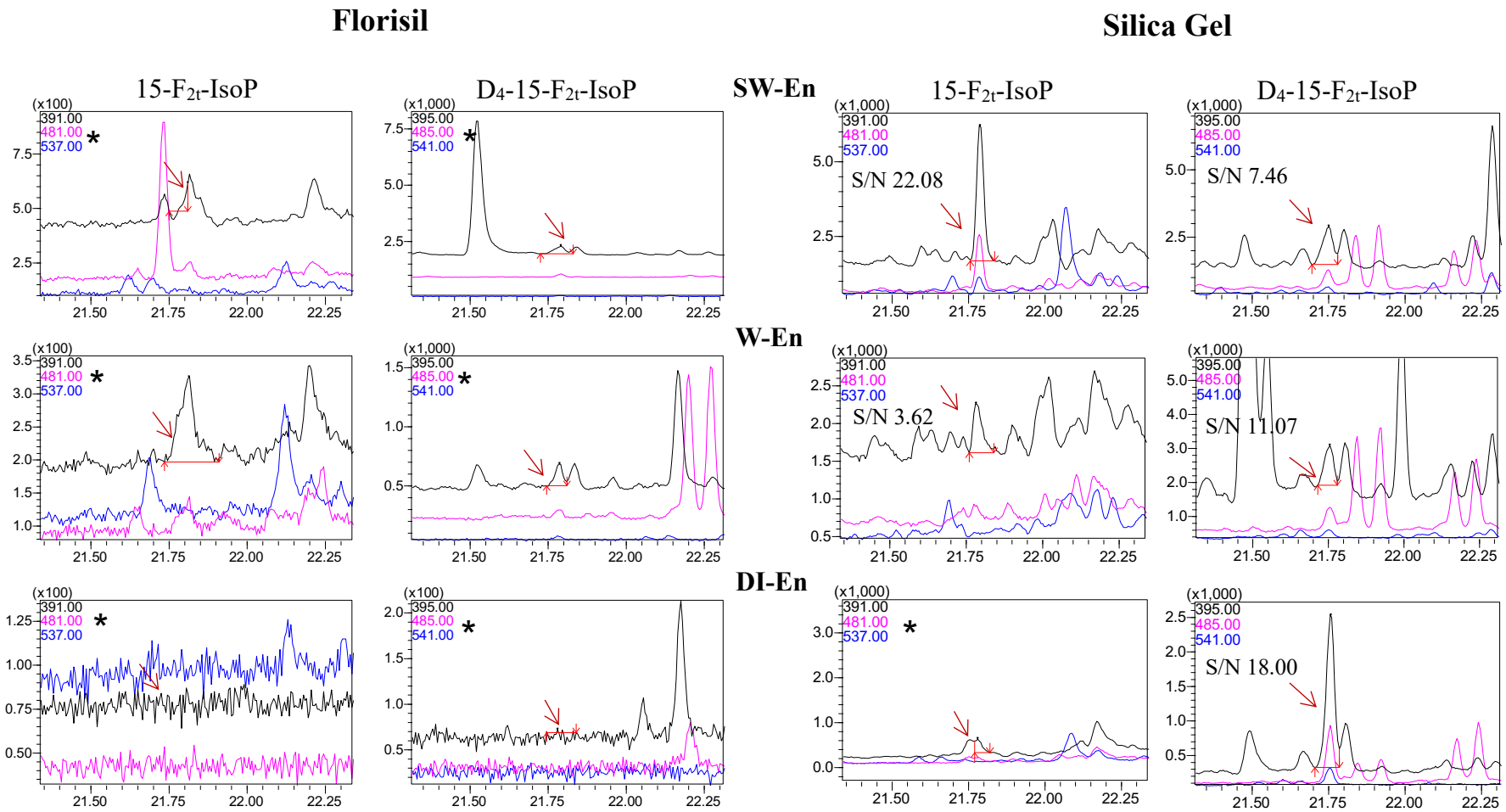

*Figure 2-9. A comparison between the chromatograms of 15-F_{2t}-IsoP and D_4-15-F_{2t}-IsoP purified through either florisil or silica gel chromatography. *Denotes results where the native or surrogate were not detected due to inaccurate ion ratios, low signal to noise ratios or interfering peaks. An arrow is used to depict the location of the IsoP peak.*

SW-En = Spiked wastewater with enzyme; W-En = Wastewater with enzyme; and DI-En = Deionised water with enzyme.

The silica gel trial was then repeated using wastewater. Extraction was conducted following the outlined methods with methanol (Section 2.4.3.2). Following their elution from the SPE cartridges, the samples were dried down under N_2 at 40°C to 0.5 mL. They were then transferred onto the pre-conditioned silica gel column with 2 x 250 µL methanol, allowed to run through, and then the analytes were eluted with 5 x 1 mL ethyl acetate:methanol (50:50, v/v) into 5 mL reacti vials. Again, these were dried down at 40°C under N_2 and derivatised with MSTFA.

The silica clean-up step was found to result in more reproducible results, while also removing some of the interferences present in the wastewater, specifically at the retention time of the analyte and surrogate (Figure 2-9, Figure 2-10). However, the significant trailing peak remained present in the majority of Bromley samples, meaning many were unable to be accurately integrated. Unfortunately, the silica gel column also a caused loss of surrogate and analyte (Table 2-8). However, overall, the recoveries were relatively consistent, other than in the Bromley samples which skewed the averages and increased the standard deviation of the results. Although there was room for the improvement of the methods, due to time constraints silica gel chromatography alongside Strata X-AW extraction was selected for sample analysis.

Table 2-8. Mean recovery for native (based on spiked samples), and surrogate (based on samples and samples spikes).

Analyte	**Mean Recovery ± SD** (%)
15-F_{2t}-IsoP	46.4 ± 27.2
D_4-15-F_{2t}-IsoP	43.1 ± 26.9

2.4.3.1 Trial 4: Liquid-liquid extraction with hexane

In a final attempt to clean-up the wastewater and provide better extractions, hexane liquid-liquid extraction was trialled near the end of sample analysis. Because hexane is non-polar, it can be used to remove other non-polar interferences, including fats and lipids, from samples. As most of the samples had already been analysed, only the influent and a small volume of effluent was available to trial this method.[124]

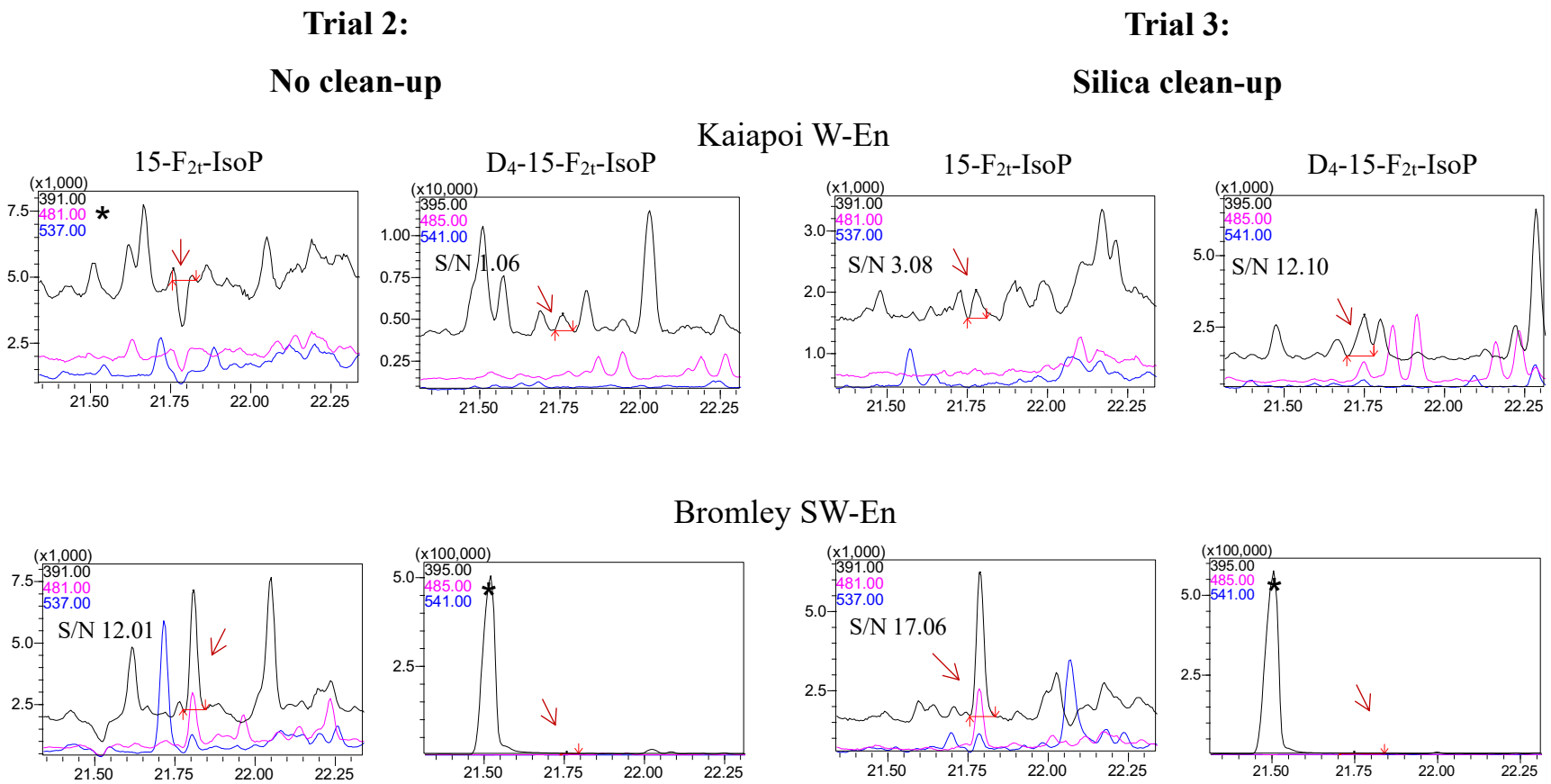

*Figure 2-10. A comparison between Trial 2 and Trial 3 chromatograms of Kaiapoi and Bromley effluent samples. *Denotes results where the native or surrogate were not detected due to inaccurate ion ratios, low signal to noise ratios or interfering peaks. An arrow is used to depict the location of the IsoP peak.*

Kaiapoi W-En = Wastewater with enzyme; Bromley SW-En = Spiked wastewater with enzyme.

Firstly, 5 mL of hexane was added to 30 mL of methanol (the eluted volume), thoroughly homogenised, and then left to allow for layer separation. However, because the ratio between the two solvents was too large, the sample had to be left overnight for only partial phase separation. Instead, a methanol:hexane ratio of 1:1 or 2:1 was determined to be the optimal volumes of each solvent which allowed for rapid density separation while also maintaining enough liquid to safely remove the hexane layer. Following elution from the extraction cartridges, the solvent was dried under N_2 to 5 mL and then a 5 mL aliquot of hexane was added. The amber glass vial was then thoroughly mixed through inversion and left to sit for 5 minutes. The upper hexane layer was then carefully removed without disturbing the methanol layer. The process was repeated with an additional 2 x 5 mL of hexane. Following this, the methanol was dried down to ~0.5 mL, and transferred into a reacti vial for derivatisation with MSTFA.

Hexane trial: GC-MS and results.

The GC-MS methods used were the same as previously mentioned (Section 2.4.3.1). The hexane clean-up technique appeared to improve spike recoveries while providing a similar clean-up efficiency to the silica gel chromatography (Table 2-9). A full comparison between the hexane and silica gel chromatography clean-up strategies could not be conducted due to a shortage of wastewater samples. Although, based on these preliminary results, it is likely that had this method been available prior to the effluent extractions, it would have further improved the recoveries and reproducibility of both the effluent and influent results. In future, it would be important to conduct repeated trials with wastewater from the same plant to determine the reproducibility of the method and the additional steps which could be undertaken to further clean-up the wastewater samples. Unfortunately, the hexane was unable to remove the interferences associated with the Bromley wastewater, which in the influent samples severely impacted the quantification of the surrogate concentrations in many samples, consequently decreasing the proportion of values which could be accepted. Table 2-10 presents a summary of the extraction and clean-up trials, specifying the optimal methods.

Table 2-9. Mean recovery for native (based on spiked samples), and surrogate (based on samples and samples spikes) effluent samples through hexane clean-up.

Analyte	**Mean Recovery ± SD** (%)
15-F_{2t}-IsoP	146.1 ± 45.8
D_4-15-F_{2t}-IsoP	56.0 ± 47.2

Table 2-10. A summary of the extraction and clean-up trials conducted for method development.

Trial	Cartridge and clean-up	Pre-condition	Drying (hrs)	Elution	Optimal method
1	Strata X and X-AW	3 x 5 mL of each of acetone, methanol and MQ.	2-3 hrs	Acetone	Strata X-AW
2	Strata X-AW	3 x 5 mL of each of acetone, methanol:acetic acid (98:2) and 1 x 5 mL MQ	2.5 hrs	Methanol or acetone	Methanol elution
3	Strata X-AW + Silica column or florisil clean-up	3 x 5 mL of each of acetone, methanol:acetic acid (98:2) and 1 x 5 mL MQ	2.5 hrs	Methanol	Silica gel chromatography
4	Strata X-AW + Hexane clean-up	3 x 5 mL of each of acetone, methanol:acetic acid (98:2) and 1 x 5 mL MQ	2.5 hrs	Methanol	Hexane

2.5 D_4-15-F_{2t}-Isoprostane surrogate and internal standard

Following the initial extraction trails, D_4-15-F_{2t}-IsoP was introduced as a surrogate to be spiked into the samples prior to extraction to determine analyte recoveries (Figure 2-11). A series of ions were selected based on those used for 15-F_{2t}-IsoP and those outlined in Bessard et al. (2001)[76] to identify D_4-15-F_{2t}-IsoP. First, m/z 395 was chosen as the quantifier ion, then m/z 485 and 541 as the qualifier ions. A MSTFA blank, 1 μg/mL derivatised D_4-15-F_{2t}-IsoP (surrogate), and 1 μg/mL derivatised D_4-15-F_{2t}-IsoP with 1 μg/mL 15-F_{2t}-IsoP (native and surrogate) were then run to ensure that there were no interferences between the MSTFA and D_4-15-F_{2t}-IsoP, or between the surrogate and the native.

Next, BPC (Bisphenol C) was introduced as an internal standard to account for derivatisation and injection efficiency. Mass to charge ratios of m/z 385 and m/z 386 and 400 were used as the quantifier and qualifier ions, respectively. A derivatisation agent blank and a 0.1 μg/mL BPC standard were run and compared to identify the retention time of the compound (17.615 minutes) and to confirm that the BPC did not cause any interferences with the surrogate or native IsoP.

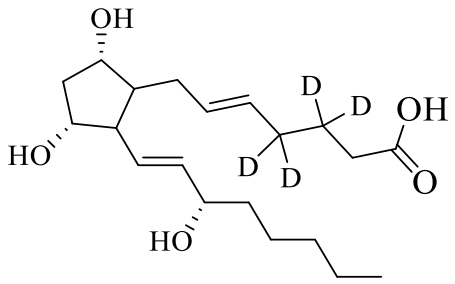

Figure 2-11. Structure of D_4-15-F_{2t}-IsoP.

Chapter 3

Optimised Sampling and Analytical Methods

3.1 Wastewater sampling: Protocol and sample collection

3.1.1 Wastewater treatment plants and sampling plan

The WWTPs were selected based on several factors which allowed for a greater representation of urban, rural and industrial waste, as each source can contribute different volumes of wastewater and classes of contaminants.[125] The different locations of these WWTPs around the Canterbury region ensured that there was a degree of variation between the size of the populations each plant serves, and the types of waste being processed (See Section 4.1). Initial communication with representatives from the WWTPs was aided by Mike Bourke from Christchurch City Council. Each plant was visited prior to sampling for safety inductions and to assess the most appropriate sites to set up the samplers.

Temporal characterisation of the four WWTPs (Bromley, Governor's Bay, Kaiapoi and Lyttleton) began in June (Figure 3-1). Both weekend and weekday 24 hour composite samples were collected to account for any potential differences in waste composition over a 7 day period. To ensure that the effluent was representative of the influent, the beginning of the 24 hour sample collection was staggered based on the retention time of the plant (Table 3-1). Following this, three WWTPs (Bromley, Kaiapoi, and Lyttleton) were to be sampled monthly for 6 months. Initially, Ashburton was also to planned to be sampled during the temporal characterisation phase. However, as a result of sampler failures, time restrictions and the long (>30 days) retention time of the plant, it was removed from the sampling plan. During September, the 6 month plan was also changed so that solely effluent samples were collected and analysed every second month (August, October and December) to ensure adequate time for sample analysis and data collaboration.

Table 3-1. The retention times of the WWTPs sampled.

WWTP	Retention time	Time delay between influent and effluent sampling
Bromley	4-5 hours	5 hours
Governors Bay	24 hours	24 hours
Kaiapoi	48 hours	48 hours
Lyttelton	24 hours	24 hours

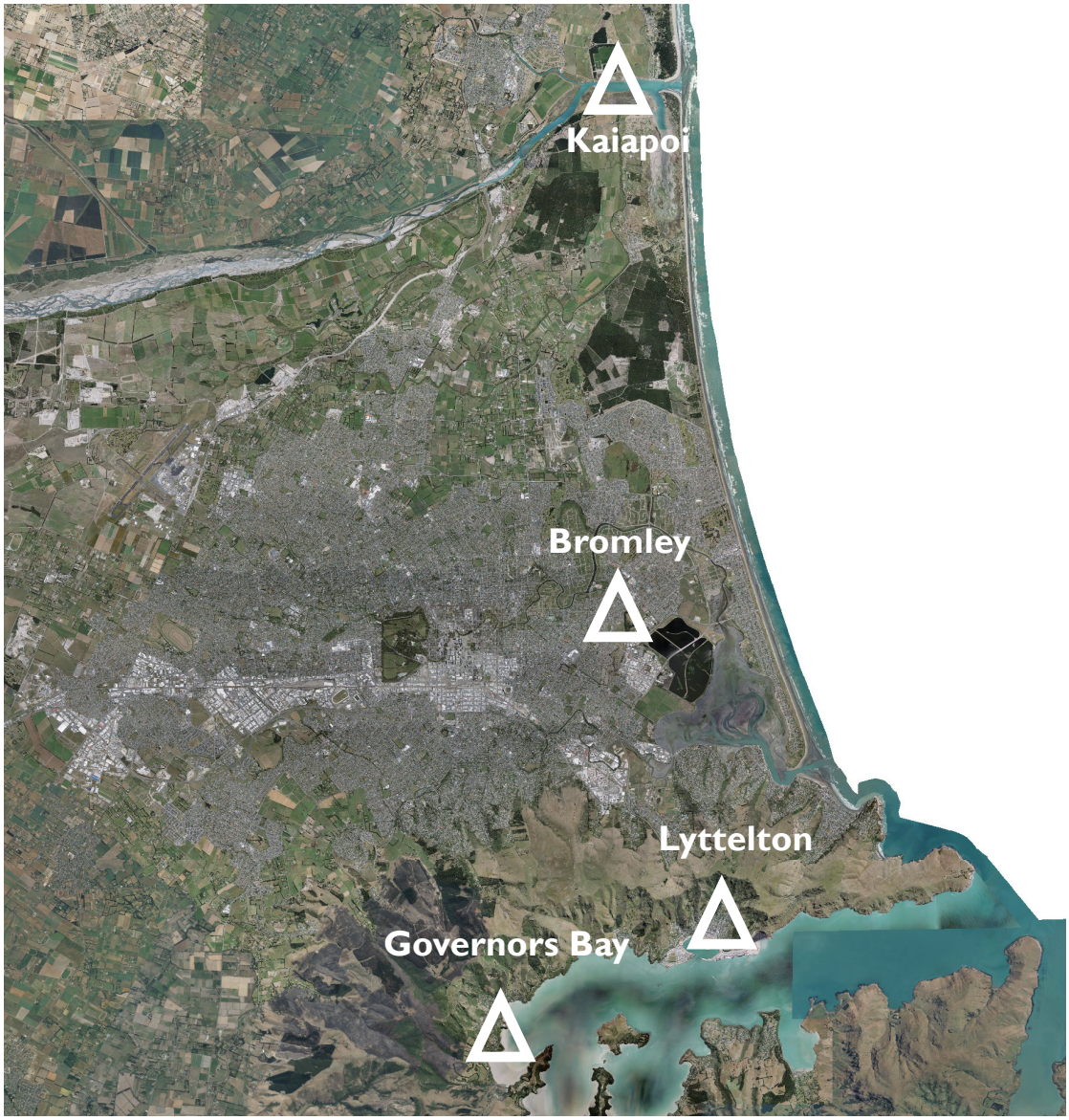

Figure 3-1. Wastewater treatment plant locations within Canterbury. Map designed on Canterbury Maps.

3.1.2 Sampler set-up

Two ISCO 3700 portable samplers, with 24 1 L bottles each, were used to collect the samples (Figure 3-2). Each site had two polytetrafluoroethylene (PTFE) hoses, one each for the influent and effluent. These were measured and cut to the nearest foot, then entered into the samplers and calibrated to ensure accurate and reproducible volumes of wastewater were collected. The hoses were replaced if they were damaged or could not be thoroughly cleaned. For each sampling event, an excel spreadsheet was produced, detailing the timing and volumes of the samples (See Appendix 1). Each sampler was set to collect 250 mL, every 30 minutes for 25.5 hours, placing three samples per bottle (750 mL). The contents of the first bottle (first 1.5 hours of sampling) was disposed of as it served to flush and prime the sampler hosing. Following unforeseen circumstances, 125 mL samples were collected every 15 minutes at the Kaiapoi WWTP to reduce sample inconsistencies resulting from the large and rapid fluctuations in the influent volumes at the plant and to increase the likelihood of a sample being collected during a state of flow.

All equipment that came in contact with the wastewater, including the sampler bottles, bucket, lids and stirrer, were soaked in Vircon for at least 24 hours following sample collection. Following this, they were rinsed 3 times with deionised water, then solvent-washed 3 times with both methanol and acetone. The sampler bottle lids were lined with aluminium foil prior to each sample collection and were cleaned alongside the bottles.

Figure 3-2. ISCO 3700 portable sampler set up at Kaiapoi wastewater treatment plant and the sampler bottles.

3.1.3 Sample processing and storage

Following sample collection, the samples were brought back to the laboratory on ice, then transferred and mixed in a stainless steel bucket. The composite samples were thoroughly homogenised, and then two aliquots of 500 mL were transferred into two solvent-washed (3x with each of methanol, acetonitrile and acetone) high density polypropylene (HDPE) bottles. The remaining wastewater (11 L) was provided to a complimentary project. The samples were immediately placed in the freezer (-20°C) until analysis. A sample blank of 1 L was also placed in the sampler for every sampling event and carried through the sampling and analytical methods in the same manner as the samples. The blank was also frozen in an HDPE bottle following the processing of the samples.

3.1.4 Health and safety

Prior to each field trip, a risk assessment was also completed and submitted to the Department Safety Officer. Due to the hazardous nature of the wastewater matrix, gloves were worn at all times. “Clean” jobs were done prior to “dirty” jobs, such as packing up the battery and sampler top unit, before putting lids onto the sampler bottles and then removing the hosing, disinfecting it with ethanol, and placing it in a bag for transport back to the laboratory. Gloves were replaced regularly and placed in a rubbish bag. Hi-visibility jackets and gumboots were worn at the WWTPs. The boots were also sprayed with ethanol before they were removed. All equipment and surfaces were regularly sprayed and wiped down with 70% ethanol. Hands were either washed then sanitised, or just the latter if no facilities were available. The samplers and samples were transported on trolleys to avoid injury, and a buddy system was used.

3.2 Sample analysis

3.2.1 Filtration and deconjugation

Sample analysis was conducted in batches of 8-9: (I) Weekend and weekday effluent; (II) Weekend and weekday influent; and (III) Bimonthly effluent samples. First, the wastewater samples and field blank were removed from the freezer and defrosted in a water bath at room temperature. Ensuring each sample was homogenised, 200 mL was then measured, filtered and acidified with acetic acid to pH 5 (Figure 3-3). An additional two aliquots of 200 mL of wastewater was prepared and processed, to serve as the sample duplicate and spike. Following

this, 1.5 mL of β-glucuronidase was added, and the samples were incubated for 2 hours at 37°C.

3.2.2 Solid phase extraction

Following incubation, the samples were allowed to cool. They were then spiked with 200 µl of a 0.1 µg/mL D_4-15-F_{2t}-IsoP surrogate solution. At the time of spiking, a comparative was also dispensed through adding 200 µL of a 0.1 µg/mL native-surrogate standard mix. The Strata X-AW cartridges were pre-conditioned with 3 x 5 mL acetone, followed by 3 x 5 mL of methanol:acetic acid (98:2, v/v) and 5 mL ultrapure water. Each cartridge was positioned over the manifold, then a 5 mL aliquot of the solvent was added (Figure 3-4). Around half of the solvent was allowed to flow through under gravity, then the taps were closed for 2 minutes to allow the solvent to saturate the SPE bed. The taps were then opened and the remaining solvent allowed to flow through. This process was repeated for each solvent aliquot. Following the addition of the ultrapure water, an additional 5 mL was added to prevent the SPE bed from becoming exposed to air before the samples had been loaded.

The samples were then run drop-wise through the cartridges under a gentle vacuum. Once the bottles were empty, the flow was stopped so that the bottles and tubing could be rinsed by passing 2 x 10 mL ultrapure water through. Following this, the cartridges were dried for 2.5 hours. For elution, the Strata X-AW cartridges were stacked over previously-used Strata FLPR cartridges, which were filled with 5 g pre-baked granular sodium sulfate, and rinsed with 3 x 5 mL acetone (Figure 3-5). The Strata X-AW cartridges were then eluted with 6 x 5 mL methanol into 40 mL amber glass vials. Each aliquot was allowed to sit on the SPE bed for 2 minutes before it was allowed to pass through under gravity. Once all of the methanol had been run through, the vacuum was left on for ten minutes to ensure all of the eluent had been collected. The samples were then prepared for silica gel chromatography (Section 3.2.2.1) or hexane extraction (Section 3.2.2.2).

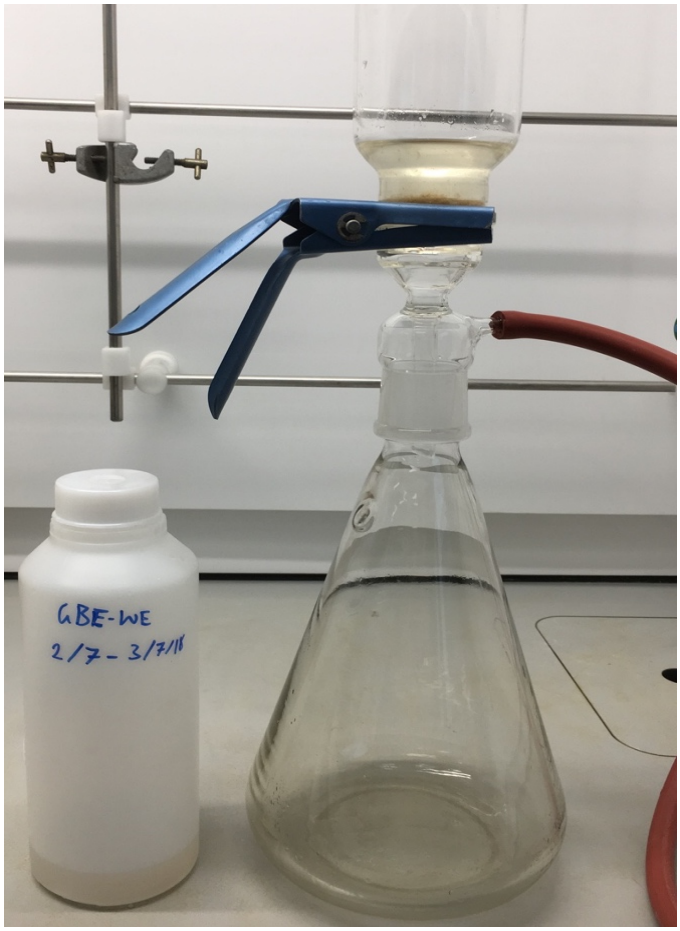

Figure 3-3. Sample filtration set-up.

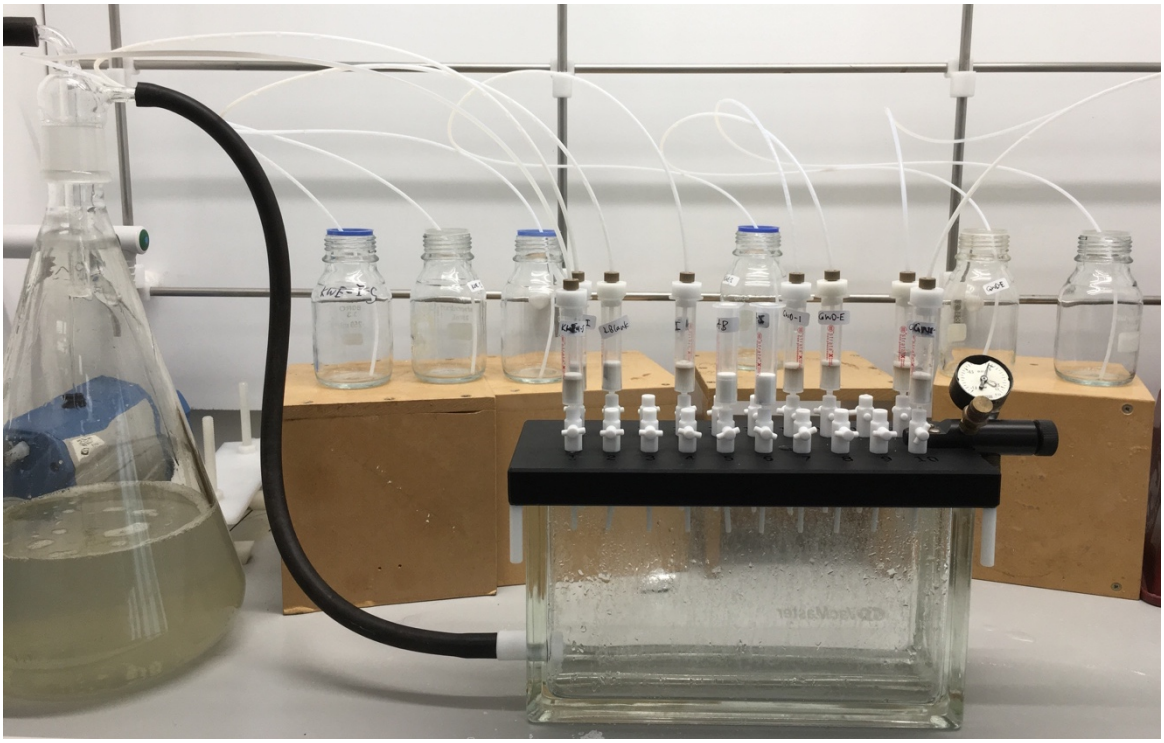

Figure 3-4. Solid phase extraction set-up.

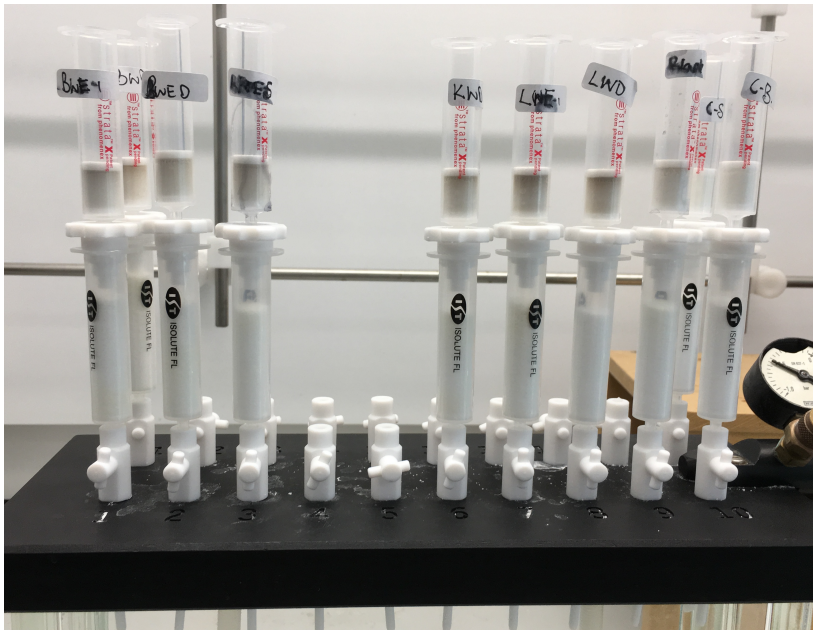

Figure 3-5. SPE cartridges stacked over sodium sulfate-packed cartridges for sample elution.

3.2.2.1 Silica gel chromatography sample clean-up: Wastewater effluent

All of the wastewater effluent samples were purified using silica gel chromatography. Following elution, the methanol was dried down to ~0.5 mL under a gentle stream of N_2 and quantitatively transferred onto the silica gel columns with 2 x 250 µL methanol. Each column was set up in a short glass pipette with glass wool packed into the bottom. Silica gel was then added on to the top of the wool until the columns were ¾ full (~0.55 g), before it was then pre-conditioned with 5 x 1 mL ethyl acetate. Each aliquot of ethyl acetate was loaded onto the silica and allowed to drip through under gravity before the remaining moisture was expunged using a pipette bulb. Once the samples had been loaded, they were eluted with 5 x 1 mL ethyl acetate:methanol (50:50 v/v). They were then dried down to ~0.5 mL before 200 µL (0.1 µg/mL) of the internal standard, BPC, was added to the samples and the comparative. Then they were all dried down for derivatisation with MSTFA (Section 3.2.3.2).

3.2.2.2 Hexane liquid-liquid extraction: Wastewater influent

The hexane clean-up technique was only used for the influent samples as the method was not trialled until after the effluent samples had already been processed and analysed. The silica clean-up technique did not provide sufficient sample purification, whereas, the hexane liquid-liquid extraction efficiently removed many of the non-polar interferences from the wastewater samples. Firstly, the eluted samples were dried down to 5 mL under N_2, then 3 x 5 mL of

hexane was added. Following the addition of a hexane aliquot, each sample was thoroughly mixed through inversion and shaking, then left to settle for 5 minutes. Following this time period, the top hexane layer was carefully removed with a glass pipette and discarded. If any of the methanol layer was collected or disturbed during this process, the pipette contents was ejected back into the vial and the layers were allowed to separate again for 5 minutes, before the process was repeated. Once the final hexane aliquot had been added, the samples were dried down to ~0.5 mL under a gentle stream of N_2, and quantitatively transferred into 1 mL reacti vials with 2 x 250 µL of methanol. These were then prepared for sample derivatisation (Section 3.2.3.2).

3.2.3 Sample derivatisation and GC-MS analysis

3.2.3.1 Synthesis of the MSTFA mix

The MSTFA was prepared by adding 95 µL of MSTFA to a reacti vial containing 3.8 mg of NH_4I and 5.67 µL 2-mercaptoethanol. The mix, and the remaining bottle of MSTFA were purged with N_2, before the mix was then vortexed and incubated at 65°C for around 2 hours, until the NH_4I was fully dissolved. Following cooling, the remaining 905 µL of MSTFA was added to the mix and vortexed. The vial was purged with N_2 following every use and stored at 4°C for a maximum of 10 days.

3.2.3.2 Derivatisation and sample transfer

Once the samples had been transferred into reacti vials, they were dried under N_2 at 40°C to ~0.5 mL, and 200 µL of the BPC (0.1 µg/mL) internal standard was added. The samples were then dried down again before 30 µL of the MSTFA mix was added. They were then mixed and incubated for 45 minutes at 65°C. Once the samples had cooled for 10 minutes, the derivatised sample was transferred into 0.1 mL GC vial inserts and 170 µL of iso-octane was then used to rinse the reacti vials, before it was also transferred into the vial inserts and mixed, making the total volume up to 200 µL.

3.2.3.3 GC-MS parameters and analysis

All method development, standard and sample analysis was conducted on a GC-MS using a Shimadzu GC-2010 Gas Chromatograph with a Shimadzu AOC-20i autosampler coupled to a Shimadzu GCMSQP2010 Plus detector. A Rxi-5Sil 30 m x 0.25 mm i.d. x 0.25 µm column (5% diphenyl/95% dimethyl polysiloxane) from Restek (1323-127) was used to separate the analyte, while Shimadzu GC-MS Solution software was used to control the instrument and

process data. A Shimadzu 10 µl syringe was used to inject 1 µl of the sample into the injection port in a splitless mode, with helium as the carrier gas. The initial oven temperature was held at 140°C for 4 minutes, then increased at a rate of 8°C/minute to 300°C. It was then increased to 310°C at 20°C/minute, and held for an additional 5 minutes. The analytes were identified based on the parameters outlined in Table 3-2, and the concentrations were confirmed based upon the calibration curve run prior to each batch of samples. To ensure consistency in the results, multiple parameters had to be met for the results in a sample to be accepted based off the criteria in US EPA method 8280A.[126] These were (I) The peak of the analyte occurred the retention time specified; (II) The ion ratios were within 20% of those specified within a standard; (III) Signal to noise ratio >2.5; (IV) Had a concentration greater than the detection limit (Section 2.3.4); and (V) Surrogate matched all of these criteria. Results were marked not detected (ND) if the values for (II), (III) and (III) were significantly different from those specified and if (I) was not met. Results which met (I), (II), and (III) but not (V) and/or (IV), were marked lower than the detection limit (LOD).

Table 3-2. Detection parameters and retention times of the analyte, surrogate and internal standard.

Compound	Quantifier ion m/z	Qualifier ion m/z	Retention time (min)
15-F_{2t}-IsoP	395	481, 537	21.815
D_4-15-F_{2t}-IsoP	391	485, 541	21.840
BPC	385	386, 400	17.615

3.2.3.4 Method detection limit

The method detection limit (MDL) was determined through the signal-to-noise approach,[127] based on the mean lowest concentrations which resulted in a S/N >2.5. This value was also checked through ensuring that sharp, clear and accurately quantifiable peaks could be identified. Due to the complexity of the matrix and the low recoveries however, a conservative approach to the MDL was also selected to ensure confidence in the results, resulting in a MDL of 8.0 ng/L. Although this number is higher than the value calculated based solely on the S/N approach, it ensured more confidence in the final results. It also meant that some results which did meet the other criteria, and produced accurately identifiable peaks, could not be included in the final results as their concentration values were lower than the MDL (1.5-8.0 ng/L).

3.2.4 Recovery and recovery correction calculations

Sample recoveries were calculated based on the surrogate concentration in extracted samples, and the concentration of the surrogate in the comparative standard (Equation 3-1):

$$R\% = \frac{C_s}{C_{Cp}} \; x\,100 \qquad \textit{Equation (3-1)}$$

R% = Recovery

C_s = Detected concentration of surrogate

C_{Cp} = Detected concentration of the surrogate in the comparative

Due to the low sample recoveries, recovery corrections were calculated to provide provisional data:

$$R_C = \frac{C_A}{R\%} \; x100 \qquad \textit{Equation (3-2)}$$

C_A = Detected concentration of analyte

R_c = Recovery corrected concentration

3.2.5 Calibration curve

A ten-point calibration (1, 2.5, 5, 10, 25, 50, 100, 250, 500 1000 ng/mL) was run with every batch of samples (Figure 3-6). It was prepared from the appropriate volumes of native and surrogate mixes. The standards were added to reacti vials, alongside 200 µL of 0.1 µg/mL BPC, and dried down under a gentle stream of N_2. They were then derivatised with MSTFA as described in Section 2.3.3. They were then transferred into GC vial inserts and analysed.

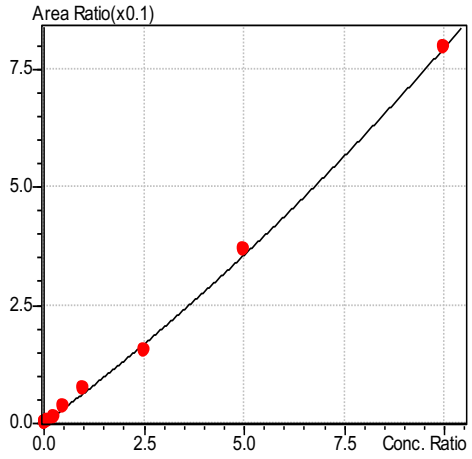

Figure 3-6. An example calibration curve.

3.2.6 Quality assurance and quality control

Glassware cleaning

All glassware was washed three times each with HPLC grade methanol, acetonitrile and then acetone. If the glassware was difficult to clean, dichloromethane was also used following the methanol. Any equipment which came in contact with the samples prior to extraction was also cleaned with Vircon, and rinsed with de-ionised water (3 x) prior to the solvent washing. The SPE equipment, including the Teflon transfer lines and end caps, were cleaned by passing methanol, acetonitrile and acetone through the tubing. The outside of the transfer line was also cleaned with each solvent.

Analytical QA/QC

Each sample batch consisted of 8-9 samples, a field blank, sample duplicate, sample spike, cartridge blank and a cartridge spike. Each of which was processed and extracted simultaneously to the samples. Prior to extraction, 200 µL of 0.1 ppm D_4-15-F_{2t}-IsoP surrogate was spiked into every solution, including the samples, field blanks and spikes. The native (200 µL of 0.1 ppm 15-F_{2t}-IsoP) was also added to the sample and cartridge spikes, and the comparative. Spike recoveries were calculated based on the concentration in the sample spike, divided by the concentration in the comparative. Any analyte detected in the cartridge blank or field blank was used to correct for analyte in the results.

Instrumental QA/QC

Several quality control practices were employed to maintain optimal instrumental analysis conditions. Prior to each batch of sample analysis and calibration standards, the rinse solvents (consisting of iso-octane, toluene and dichloromethane) and septas were changed. The injection needle was also thoroughly cleaned with a dichloromethane:methanol (95:5, v/v) mix, to ensure there were no residual crystals present which can form as a result of the derivatisation agent. Before and following each sample injection, the syringe was programmed to rinse three times in each of the rinse solvents. Duplicates were analysed every ten samples to ensure that there was no reduction in signal. At the beginning of each batch, an iso-octane blank was run. While following a sample sequence, three iso-octane blanks were run to flush any volatiles from the system which may have accumulated over the course of the batch. The results of these blanks were checked after the run to ensure that the background noise was at an acceptable level, so as not to cause any interferences. An iso-octane blank was also run half-way through a sample sequence to flush the instrument. Whenever the glass liner was changed, multiple standards and environmental samples were injected and run to ensure that the active sites within the

injection port were stabilised. Tuning files were also run to ensure that the instrument was operating under optimal parameters. Following an instrument tune, a calibration curve was formed, and the retention times and ion ratios of the analytes were confirmed to still be accurate or adjusted accordingly

Chapter 4

Survey of Canterbury wastewater treatment plants

4.1 Characterisation of the wastewater treatment plants

A series of WWTPs were surveyed to determine the concentrations of 15-F_{2t}-IsoPs present in their wastewater (Section 3.1), following the design and validation of an analytical method to enable their detection (Chapter 2). This survey was conducted to investigate whether WWTPs could represent a source of IsoPs to the environment where they may act as potential emerging contaminants (ECs).[18, 54] The WWTPs sampled in this research were selected to reflect varying treatment processes, flow rates, size and degree of urbanisation of the catchment populations, and volumes of groundwater infiltration (Table 4-1). Each of these factors has the potential to determine the concentrations of contaminants,[128, 129] including IsoPs, present in the influent, as well as their rate of removal. The flow and population data presented in Table 4-1 are average values, which are unlikely to reflect the daily or seasonal fluctuations,[130] as multiple factors can cause variations in local population statistics and wastewater flows. In Canterbury specifically, a series of earthquakes in the region damaged the sewage pipes, increasing groundwater infiltration into the wastewater network.[131] Following high rainfall events, increased wastewater influent dilution can occur, especially in the Bromley and Lyttelton WWTPs.[131] Sampling during high rainfall events was avoided when possible, however higher-than-average flows can remain for the following days. Daily commute between different areas can also alter human-waste inputs which may consequently alter the volume of human biological waste entering the plants, although the influence of this was expected to be minimal.[132]

4.2 Isoprostane concentrations detected in the survey of Canterbury wastewater treatment plants

The provisional survey results are presented in batches of sample extraction and analysis. This is to account for: (I) the differences in sample purification methods for the effluent (silica gel chromatography, see Section 3.2.2.1) (Table 4-2 and 4-3) and influent samples (hexane liquid-liquid chromatograph, see Section 3.2.2.2) (Table 4-4); and (II) the weekend versus weekday (Table 4-2), and bimonthly (Table 4-3) temporal characterisation of IsoP concentrations in wastewater effluent.

Table 4-1. Characterisation of the WWTPs surveyed in this research. Note: Population, volume treated and daily flow data are approximate values.[133]

	Bromley	Kaiapoi	Lyttelton	Governors Bay
Population	368,000	10,700	3137.2	646.8
Volume treated (m^3/yr)	68,237,480	1,621,651	348,521	73,444
Daily flow (m^3/d)	186,952	4,419	828	176
Treatment type (In process order)	- Screen to trap larger material and grit - Sedimentation tanks to remove heavy organic matter (which is then digested) - The liquid waste is then pumped into the top of trickling filters, allowing the bacteria to consume the nutrients in the wastewater. - Aeration tanks - Clarifiers (to allow bacteria slime to settle out of the liquid) - Oxidation ponds - Ocean outfall[134]	- Screening - Aeration basin to reduce organic load - Oxidation pond - Infiltration wetland - Rangiora effluent then enters the infiltration wetland, mixing with the Kaiapoi effluent, before it all passes through a smaller, planted wetland - Ocean outfall - A UV disinfection system is also used when bacteria levels are high.[135]	- Screening - Extended aeration - UV disinfection - Sludge dewatering - Ocean outfall[136]	- Screening to remove large matter - Extended aeration - UV disinfection - Ocean outfall[136]
Ratio of wastewater type	30% groundwater infiltration 10% industrial waste 60% domestic waste	Some infiltration Considered mostly domestic waste	>90% domestic waste Some groundwater infiltration	>90% domestic waste

Table 4-2. Batch 1 results for the analysis of weekend and weekday effluent samples. The table displays the concentrations of the analyte (15-F_{2t}-IsoP), the deuterated surrogate (D_4-15-F_{2t}-IsoP), and the analytes recovery corrected concentration (R_c) (ng/L).

Analyte	Blank	Lyttelton			Bromley		Kaiapoi		Governor's Bay	
		LWE-E	LWD-E	LWE-E^{D*}	BWE-E	BWD-E	KWE-E	KWD-E	GBWE-E	GBWD-E
15-F_{2t}-IsoP	ND	LOD	8.2	LOD	ND	ND	ND	LOD	ND	LOD
D_4-15-F_{2t}-IsoP	36.1	44.0	52.6	68.3	35.7	LOD	LOD	82.9	LOD	54.4
R% D_4-15-F_{2t}-IsoP	28.6	33.3	41.7	54.1	28.3	LOD	LOD	65.8	LOD	43.1
R_C 15-F_{2t}-IsoP	ND	LOD	19.6	LOD	ND	ND	ND	LOD	ND	LOD

*D = Duplicate
R% = Recovery; L = Lyttelton; B = Bromley; K = Kaiapoi; GB = Governor's Bay; WE = Weekend; WD = Weekday; and E = Effluent samples.

Table 4-3. Batch 2 results for the analysis of the bimonthly (August, October and December) effluent samples. The table displays the concentration of the analyte (15-F_{2t}-IsoP), deuterated surrogate (D_4-15-F_{2t}-IsoP), and the analytes recovery corrected concentration (R_c) (ng/L).

Analyte	Blank	Lyttelton				Bromley			Kaiapoi		
		LE-A	LE-O	LE-D	LE-D^{D*}	BE-A	BE-O	BE-D	KE-A	KE-O	KE-D
15-F_{2t}-IsoP	ND	LOD	10.3	10.7	17.3	15.6	9.2	LOD	24.5	ND	9.17
D_4-15-F_{2t}-IsoP	64.5	18.40	39.5	41.8	35.7	56.6	29.5	LOD	85.4	LOD	23.6
R% D_4-15-F_{2t}-IsoP	55.9	LOD	34.3	36.3	30.9	49.1	25.6	LOD	74.1	LOD	20.4
R_C 15-F_{2t}-IsoP	ND	7.5	29.9	29.6	55.9	31.7	35.9	LOD	33.1	ND	44.9

D* = Duplicate
R% = Recovery; L = Lyttelton; B = Bromley; K = Kaiapoi; GB = Governor's Bay; E = Effluent; A = August; O = October; and D = December samples.

Table 4-4. Batch 3 results for the analysis of the weekend and weekday influent samples. The table displays the concentrations of the analyte (15-F_{2t}-IsoP), deuterated surrogate (D_4-15-F_{2t}-IsoP), and the recovery corrected concentration of the analyte (R_c) (ng/L).

Analyte	**Blank**	**Lyttelton**		**Bromley**			**Kaiapoi****	**Governor's Bay**	
		LWE-I	**LWD-I**	**BWE-I**	**BWD-I**	**BWD-I^{D*}**	**KWD-I**	**GBWE-I**	**GBWD-I**
15-F_{2t}-IsoP	ND	19.9	LOD	12.6	ND	ND	22.9	LOD	LOD
D_4-15-F_{2t}-IsoP	76.1	76.9	LOD	20.2	LOD	LOD	67.6	LOD	LOD
R% D_4-15-F_{2t}-IsoP	59.7	60.3	LOD	15.8	LOD	LOD	53.0	LOD	LOD
R_C 15-F_{2t}-IsoP	ND	33.2	LOD	79.9	ND	ND	43.2	LOD	LOD

*D = Duplicate

**Due to a sampler issues, Kaiapoi weekend influent was not analysed

R% = Recovery; L = Lyttelton; B = Bromley; K = Kaiapoi; GB = Governor's Bay; WE = Weekend; WD = Weekday; and I = Influent samples

Due to the low recoveries of the methods (Table 2-8), the tables display both the recovery-corrected (R_c) and non-recovery corrected concentration data. The inclusion of the recovery-corrected data was important to more accurately establish the concentrations of IsoPs which may be present in wastewater, particularly for the effluent samples purified through the silica gel, which had the lowest recoveries (mean 46%) and clean-up efficiency. The results were only deemed acceptable if they met the specified criteria outlined in Section 3.2.3.3. Unfortunately, many results were marked as LOD, due to the deuterated surrogate not meeting the specified criteria, even though the analyte did. Further sample clean-up trials, as well as running all of the effluent samples through the hexane purification technique, may improve the results in future.

In the survey, 15-F_{2t}-IsoPs were detected at three of the four WWTPs, in both the influent and the effluent. No IsoPs were detected at the smallest WWTP, Governor's Bay, which may be due to a combination of the low population and the need for more sensitive analytical methods. Across the plants, the recovery-corrected concentrations range from ND-79.9 ng/L and ND-55.9 ng/L in the influent and effluent, respectively. Although 15-F_{2t}-IsoPs have been detected in the survey of the WWTPs, due to the provisional nature of the data, further investigation is required. In future, additional method development, and the analysis of a greater number of samples is necessary to more accurately determine any definitive trends in IsoP concentrations.

4.2.1 Trends between plants

Given that IsoPs are endogenously produced compounds, it was hypothesised that WWTPs with higher mammalian waste inputs (i.e. domestic or agricultural waste), would also have greater IsoP concentrations present in their wastewater. The WWTPs surveyed in this study primarily process domestic waste, with only Bromley processing a significant proportion of other (industrial) waste (Table 4-1). At this stage, no trends related to the proportion of mammalian inputs can be identified. Additionally, no seasonal associations or trends were identified based on the bimonthly sample results within each WWTP (Table 4-2). However, more sensitive analytical methods may be required for future certainty.

The preliminary results in Table 4-4 indicate that when detected, there are greater (recovery-corrected) concentrations of 15-F_{2t}-IsoP in the influent of the larger WWTPs such as Bromley and Kaiapoi (76.9 and 43.2 ng/L), compared to Lyttelton and Governor's Bay (33.2 ng/L and

LOD) (Table 4-4). However, a greater range of data is required to determine the significance and reproducibility of these values, as with improved data collection, a more robust assessment of IsoPs as ECs can be made.

4.2.2 Removal efficiencies of isoprostanes from wastewater

Higher concentrations of 15-F_{2t}-IsoPs were detected in the influent (ND-79.9, mean 17.4 ng/L) compared to the effluent (N.D-55.9, mean 13.2 ng/L), which indicates that WWTP processing reduces the concentrations of IsoPs present in wastewater (Table 4-5). However, although the retention times of the of WWTPs are 4-48 hours (Table 3-1), 15-F_{2t}-IsoPs were still detected in the effluent, which infers that IsoPs are stable in wastewater for greater than 24 hours. This result is consistent with previous stability studies, and suggests that IsoPs may also remain stable in environmental matrices.[106] It is also important to note that the concentrations detected in the influent and the effluent are not fully representative of what may actually be present in wastewater, as further method development is required to improve recoveries, remove interfering substances and lower the detection limit, which would improve the quantification of the IsoPs.

Table 4-5. Comparison between the influent and effluent, weekend and weekday, 15-F_{2t}-IsoP concentrations (raw and recovery-corrected concentrations, ng/L).

Wastewater sample (ng/L)	**Lyttelton**		**Bromley**		**Kaiapoi**		**G. Bay**	
	LWE	LWD	BWE	BWD	KWE	KWD	GBWE	GBWD
Influent IsoPs	19.99	LOD	12.63	NQ	N/A	22.9	LOD	LOD
Effluent IsoPs	LOD	8.2	ND	ND	ND	LOD	ND	LOD
R_C Influent IsoPs	33.2	LOD	79.9	NQ	N/A	43.2	LOD	LOD
R_C Effluent IsoPs	LOD	19.6	ND	ND	ND	LOD	ND	LOD

R% = Recovery; L = Lyttelton; B = Bromley; K = Kaiapoi; GB = Governor's Bay; WE = Weekend; and WD = Weekday

An additional factor which may be determining the removal rates of IsoPs from wastewater may be their physical sequestration onto solid-phase organic matter and sludge.[137] The phase partitioning of IsoPs is not currently understood and additional research is required to determine their partition coefficient (K_d) which may be used to derive these phase interactions.[137] Any possible phase partitioning may be dependent upon whether the IsoPs are in their glucuronide-conjugate forms, as conjugation commonly increases the polarity and hydrophilicity of molecules which can decrease their partitioning to the solid-phase.[138, 139] Alongside removing IsoPs from the liquid phase in the wastewater, sorption could also be decreasing the concentrations of IsoPs available for detection using the described analytical method. Additionally, these sorption processes and interactions may also affect the behaviour of IsoPs following their environmental discharge, and will consequently need to be further investigated.

4.2.1 Weekend versus weekday isoprostane concentrations

Elevated IsoP concentrations (including 15-F_{2t}-IsoP) are detected in the blood and urine in response to drug and alcohol consumption.[140, 141] As a result, it was anticipated that greater 15-F_{2t}-IsoP concentrations may be present in the weekend compared to the weekday samples, due to an increase in recreational substance abuse.[142-144] Based on these preliminary results, there is no discernible link between 15-F_{2t}-IsoP concentrations and the period of the week (Table 4-5). For example, while there appears to be a temporal trend at the Lyttelton WWTP based on the greater IsoP concentrations detected in the weekend (R_C, 33.1 ng/L) compared to the weekday (LOD) influent samples, the effluent concentrations contradict this result, with higher concentrations detected in the weekday (R_C, 19.6 ng/L) compared to the weekend (LOD) effluent samples (Table 4-5). As a result, more reliable data, collected at a larger scale may be required to determine if there is a temporal difference in IsoP concentrations. Ryu et al (2016)[109] were also unable to identify a temporal trend in IsoP concentrations in European WWTP wastewater. The absence of any observed differences between weekday and weekend IsoP concentrations could be due to the difficulty of collecting representative wastewater samples throughout the treatment plants. In WWTPs that serve larger urban areas such as Bromley, untreated wastewater can pass through multiple pumping stations prior to reaching the WWTP.[145] As a result, it can take multiple days for the wastewater to enter the WWTP, and the influent being sampled may not reflect any temporal changes.

4.3 Isoprostane loading and comparison to biological matrices

Greater 15-F_{2t}-IsoP loads were observed in wastewater influent by Ryu et al (2015 and 2016)[106, 109] and Santos et al (2016)[108] in association with increased urbanisation. A population normalisation equation, adapted from Ryu et al (2015 and 2016)[106, 109] was used to determine the predicted IsoP loads in Canterbury influent based on the recovery corrected concentrations (Equation 4-1, Table 4-6).

$$\textbf{IsoPs load per day per 1000 people (mg/d/1000 people)} = \left(\frac{((C_{IsoP}/1000)\ \text{mg/m}^3\ x\ \text{Daily flow m}^3\text{/d})}{\text{Population}} \right) x\ 1000$$

Equation (4-1)

C_{IsoP} = Recovery-corrected concentration of 15-F_{2t}-IsoP in the influent

The IsoP loads calculated in Table 4-6 represent approximate values due to the provisional nature of the results. The normalised values indicate that the more urbanised Bromley WWTP (which serves the majority of Christchurch City), has the greatest IsoP load (40.6 mg/d/1000 people) in comparison to the other plants (Table 4-6), which follows a similar trend to those of Ryu et al (2016).[109] Despite this, the values found in this research are an order of magnitude greater than those calculated by Ryu et al (2016).[109] This may be the result of using the recovery-corrected concentrations, and may be amended through conducting a reassessment using improved analytical methods.

One explanation for the link between urbanisation and the IsoP loads identified in this study (as and in European cities), may be that urbanised communities have an increased ratio of individuals suffering from elevated oxidative stress levels. For example, oxidative stress and mental impairment are elevated in urban versus rural aging communities.[146] Alongside the ever-increasing size of elderly populations, age contributes to elevated IsoP concentrations, both directly through increasing oxidative stress, and indirectly due to the link between age and an increased incidence of disease, such as Alzheimer's and cardiovascular disease (CVD).[147, 148 149] It is also common for urbanised areas to have a number of medical facilities (e.g. hospitals and procedural clinics), that are representative of localised areas of chronically or acutely unhealthy individuals. For example, the Bromley WWTP serves at least three hospitals and multiple medical facilities, whereas the other plants only serve smaller healthcare

providers. Consequently, it is expected that medical facilities could be significant contributors to IsoP concentrations in wastewater, as many ailments or diseases which warrant admission to hospitals are also linked to oxidative stress.[71] In addition to this, urbanisation is linked to higher rates and risks of disease incidence, with air pollution especially, believed to contribute to oxidative stress and respiratory diseases such as asthma.[150, 151] Each of these factors may at least partially account for the relationship between IsoPs and urbanisation. Moreover, the discharge of these compounds into urban environments is likely to have adverse physiological impacts on the biota living in these areas. Especially, given their pre-existing elevated states of oxidative stress, effectuated in response to increased exposure to anthropogenic pollutants in these urban habitats.[152]

Table 4-6. A comparison between daily isoprostane loads per 1000 individuals, based on Canterbury survey data and literature values from Ryu et al (2016).[109]

Wastewater sample ID	**Population**	**Wastewater flow** (m^3/d)	**Daily load per capita** (mg/d/1000 people)
Canterbury Survey*			
BWD-I	368,000	186,952	40.6
KWD-I	10,700	4419	17.8
LWE-I	3137.2	828	8.8
Literature values[109]			
Bristol	886,650	202,224.3	2.6
Brussels	953,987	265,681.8	2.8
Castellon	180,690	39993.5	2.6
Copenhagen	531,000	143,287.0	4.9
Hamar	55,000	50,695.1	5.5
Milan	1,100,000	493,085.71	5.0
Oslo	580,639	274,588.6	10.0
Stavanger	240,000	229,706.6	8.0
Tromsø	20,000	18,220.8	7.7
Utrecht	300,000	46,425.0	2.5
Zurich	410,000	190,278.0	7.8

*Using recovery corrected concentrations

L = Lyttelton; B = Bromley; K = Kaiapoi; GB = Governor's Bay; WE = Weekend; and WD = Weekday

4.3.1 Wastewater versus biological isoprostane concentration comparisons, and their relevance as emerging contaminants

The IsoP loads determined in this study (8.8-40.6 mg/d/1000 individuals), and by Ryu et al (2016)[109] are significantly larger than those previously calculated for urinary IsoP loads (0.1-5 mg/day/1000 individuals).[109] This latter value was hypothesised based on the daily urinary excretion data, which is considered to be between 100-5000 ng/day/person, depending on the isomer, with most in the range of 1000-2000 ng/day/person.[17, 153] The discrepancies between these per capita loads, may be due to the fact that the hypothesised range is based upon IsoP concentrations detected in the urine of healthy individuals, and does not take into account that a proportion of the population may be under elevated levels of oxidative stress, which could be skewing the data collected in this study and Ryu et al (2016)[109] to higher IsoP concentrations.

The recovery-corrected concentrations of 15-F_{2t}-IsoP detected in wastewater influent (ND-79.96 ng/L) and effluent (ND-55.96 ng/L), are lower than the concentrations excreted in the urine of healthy individuals 100-1200 ng/L.[154, 155] This was to be expected, due to the breakdown and dilution of these molecules in wastewater. Despite this, these concentrations are still within the range of those detected in blood (0.035-45.1 ng/L),[17, 71] suggesting that they may be biologically-relevant concentrations, potentially capable of interacting with biomolecules within exposed organisms.

Based on the presence of 15-F_{2t}-IsoPs in wastewater effluent, there is the potential for these molecules to be released into the wider environment. Although increased dilution of these concentrations is likely to occur, given that 15-F_{2t}-IsoPs have been found to remain stable in wastewater,[106] they may also remain stable in environmental matrices long enough to result in pseudo-persistence where, like other known ECs (e.g. endocrine disruptors and PPCPs), they may also represent ecotoxicological risks.[4, 156] Previous assessments of other ECs in wastewater and surface water, have demonstrated that that even when only relatively low concentrations of contaminants are detected in wastewater effluent, they can still be discharged into the wider environment and pose a threat to ecosystems (Table 4-7).[4]

Table 4-7. The concentrations of other known emerging contaminants in wastewater effluent and nearby surface water.

Contaminant	**Effluent** (ng/L)	**Surface water** (ng/L)
17α-estradiol[157, 158]	0.1-5.0	0.1-3.0
17β-estradiol[157, 158]	0.4-12.0	0.3-2.8
Androstenedione[158]	4.5-12.0	3-9.0
Androsterone[158]	N.D-4.3	ND
Bisphenyl A[159, 160, 161]	35.0-86.0	<6.0-34.0
Butylparaben[159, 160]	<1.0	<0.3-6.0
Estrone[157, 158]	0.1-47.0	0.1-3.5
Methyl paraben[159, 160]	<3-50	<0.3-68
Testosterone[158]	0.7-1.2	0.1-0.5
Triclosan[159, 160, 161]	25.0-200.0	5.0-48.0

ND = Not detected

These preliminary results provide a representation of IsoP concentrations in Canterbury wastewaters. Considerable work was conducted to improve the extraction and detection methods of IsoPs in wastewater, to contribute to this area of research. As such, these provisional results provide a platform from which further investigation may conducted

Chapter 5

Conclusions, limitations and recommendations

5.1 Summary of research and findings

5.1.1 Summary of research

The objectives of this thesis were to; (I) develop a GC-MS analytical method to measure IsoPs in wastewater; and (II) survey the concentrations of 15-F_{2t}-IsoP in the influent and effluent collected from WWTPs around the Canterbury region, to determine if wastewater may represent a source of IsoPs to the environment. This is the first known study to analyse IsoPs in wastewater from an EC perspective, and although more work is required to refine the extraction and detection techniques, significant steps have been made utilising and adapting the range of methods already available in the literature.

Most importantly, to enable the analysis of IsoPs in wastewater, novel extraction and derivatisation techniques were established. Initially, DIMETRIS, a recently developed derivatisation agent was trialled (Section 2.3). Extensive efforts to identify the ions representative of the IsoP were unsuccessful. As this derivatisation agent had previously only been used for the esterification of aromatic ring-containing compounds, the lack of success may have been a result of 15-F_{2t}-IsoP and DIMETRIS being incompatible.[117, 118] Next, a combination of BSTFA with PFB-Br, which are commonly used for the analysis of IsoPs,[94, 102, 120] were trialled and were also unsuccessful (Section 2.3.2). The reasons behind this were unable to be discerned, and future investigation is required. Finally, derivatisation with MSTFA was developed and resulted in the reliable detection of 15-F_{2t}-IsoP.

Subsequently, a method was developed for the extraction of IsoPs from wastewater with a β-glucuronidase deconjugation pre-treatment, followed by SPE on Strata X-AW cartridges. Both silica gel chromatography and hexane liquid-liquid extraction were validated for sample purification, although the latter was more efficient with improved analyte recovery (Section 2.4). These methods were then combined to allow for the analysis of IsoPs in wastewater samples. The wastewater samples were collected from four WWTPs around the Canterbury

Region. Both weekend and weekday, influent and effluent samples were collected to determine if there were any differences in the concentrations of IsoP depending on the period of the week. Finally, effluent samples were collected bimonthly over 6 months, to investigate the presence of any seasonal trends.

5.1.2 Summary of findings: Isoprostanes as potential emerging contaminants?

Isoprostanes have been postulated to act as potential contaminants by initiating a cycle of oxidative stress throughout ecosystems (Section 1.5).[18] The results of this research indicate that IsoPs are present in the wastewater of Canterbury WWTPs, and that these compounds are not sufficiently removed during the treatment process (Section 4.2.2). Consequently, suggesting that the discharge of effluent may represent a source of these bioactive compounds to the environment, where they may trigger a cycle of oxidative stress, amplifying it throughout the receiving ecosystems.[54] Although no temporal trends could be observed, additional research is required to refine a more sensitive analytical technique and achieve improved quantification. Further investigation is also required to ascertain their roles as ECs, as no definitive conclusion can be reached until toxicology research and fate studies of these molecules in surface waters have been conducted.[12, 54]

These preliminary results provide a representation of the IsoP concentrations present in wastewater, and an indication of those which may be released into the wider environment. Based solely on these concentrations, and without a detailed understanding of their environmental fate, IsoPs may be ECs of concern, especially considering that their loads in wastewater are linked to the degree of urbanisation of the surrounding population. In many cities, wastewater is discharged into nearby freshwater ecosystems, such as rivers and streams.[162] Due to the urban nature of these waterways, even without wastewater inputs, they are often in already degraded states. This state, termed the *Urban Stream Syndrome* (USS),[163] comprises various symptoms including altered hydrology, poor species diversity, high sediment loads, and elevated contaminant and nutrient concentrations that adversely affect the overall quality and sustainability of urban freshwater ecosytems.[163] The release of additional contaminants through wastewater could have untold detrimental effects on these already degraded ecosystems. If IsoPs do prove to be contaminants of concern, they may be placing additional pressure and stress on the organisms within these ecosystems. Especially, if they are capable inducing of oxidative stress and other pathological processes.

Endogenous compounds excreted by humans and other mammalian species are likely to become greater concerns as a consequence of rising populations. Rising human and other mammalian (e.g. agricultural livestock) population densities may mean that excreted endogenous compounds will continue to become a growing concern. Increasing population density will place greater strains on wastewater networks, while also augmenting the volume of biological waste which will be released into the environment.[164] The accidental release of untreated wastewater through wastewater overflows may also result in the discharge of wastewater directly into waterways. Resulting in the highly concentrated release of contaminants, potentially including IsoPs, and triggering a range of biological impacts which may alter the natural physiological functions of the aquatic biota present in these receiving ecosystems.[54]

5.2 Research limitations and recommendations

The two primary limitations of this study were the low recoveries of the extraction method and the inefficient sample clean-up. Further method development is required to attain improved extraction and purification procedures, which may allow for lower detection limits and better quantification of the IsoPs in wastewater. Alternative clean-up procedures may include trialling different solvents (e.g. alternative mixtures of solvents or those with different polarities) through silica gel chromatography, alumina clean-up and gel permeation chromatography (GPC). These clean-up procedures are commonly used for the purification of environmental matrices for the analysis of other ECs, and may also be applicable for IsoP analysis.[165, 166] As a subset of size exclusion chromatography, GPC in particular, may be a useful technique to separate the target analyte from the interfering substances in the matrix.[167] Briefly, the whole sample is loaded and eluted from the GPC column. The larger molecules, including fats (which are considered to be the primary interferences for IsoP detection in this method), are eluted in an earlier fraction which is then discarded, and only the fraction containing the analyte is collected, as a result hopefully improving IsoP quantification.

Numerous SPE procedures are also described in literature, most of which were unable to be trialled due to the time constraints of this project. Altering the pre-conditioning solvents, adding wash steps, changing the elution solvent and testing different SPE cartridges are all alternatives which may improve the extraction of the IsoPs from the matrix and the

recoveries.[168] Introducing a hexane wash step to the current extraction procedure may also improve the removal of interferences from the sample (in particular, the non-polar fats and lipids).[169] Immunoaffinity chromatography is another effective method for sample preparation and purification,[104] which based on the methods of Ryu et al (2015),[106] provides efficient clean-up of wastewater for IsoP analysis, and may be trialled prior to GC-MS.

Gas chromatography coupled to mass spectrometry was selected for the purpose of this research as it was the most sensitive, low cost method available. However, LC-MS may be used as an alternative analytical technique as it has the potential to improve the quantification of IsoPs, while enabling simpler sample preparation through eliminating the need for the derivatisation reaction, which can otherwise decrease analyte recoveries.[170] However, an issue with LC-MS is its low sensitivity in comparison to GC-MS.[96] This may mean that tandem mass spectrometry will be required to achieve lower method detection and quantification limits.[170]

An additional limitation is the small-scale nature of the survey of WWTPs. Implementing a larger sampling program would enable a greater number of samples to be collected on a temporal (i.e. more samples per plant over time) and spatial (i.e. more WWTPs) scale. This would be constructive to determine how different circumstances (i.e. the population, urbanisation, treatment processes and flow rates) affect the concentrations present. This may help provide more reliable and robust data, from which any trends in IsoP concentrations may be more accurately determined. The sampling of WWTPs with lower groundwater influences may also be beneficial as it will ensure that the primary contributing factors influencing IsoP concentrations are the population, the proportion of domestic, agricultural and industrial waste, and the degree of urbanisation of the surrounding area.

Wastewater land application and the use of biosolids as soil amendments represent alternative environmental fate pathways for many contaminants.[171] Further consideration of the behaviour of IsoPs may be required due to the increasing incidence of these wastewater disposal techniques. Additionally, the sampling of dairy shed effluents may be of interest, to determine if agricultural activities may also represent a source of these compounds to the environment,

which, combined with the high nutrient and pathogen loads,[172, 173] may be placing additional pressures on ecosystems, especially the sensitive freshwater streams of New Zealand. Alongside the development of a more robust analytical method, the one developed in this thesis may still be applicable for the analysis of other, less complicated environmental matrices. The complexity of the wastewater matrix was one of the primary issues limiting IsoP quantification in this study. As a result, the method may be more successful for the analysis of surface water (e.g. fresh and saltwater), in preliminary investigations regarding the presence of IsoP molecules in the wider environment.

5.3 Final conclusions

The discharge of wastewater effluent from WWTPs represents a continuous source of contaminants to the environment. Such discharges may serve as a pathway for the entry of biologically-active IsoP molecules to aquatic environments. As the deleterious mechanisms of oxidative stress are conserved between species, the exposure of aquatic organisms to IsoPs may result in the initiation of a cycle of oxidative stress. This may subsequently translate to the spread of oxidative stress throughout the localised environment, much like a contagious disease, and putatively lead to the excretion of additional IsoPs and the continuation of the cycle.

This body of work successfully contributes to the current research regarding the concentrations of IsoPs in WWTPs and their potential roles as ECs. To further this area of research, more work is required to refine existing extraction and detection methods, in order to reliably assess the role of IsoPs as contemporary ECs. Comprehensive surveys on their environmental concentrations, alongside a series of ecotoxicological and biological assays are also required. Simply establishing that IsoPs may be entering aquatic environments does not constitute as tangible proof that they will pose a threat, and this environmental fate data is necessary to begin to evaluate their risks as ECs. The discharge of wastewater into sensitive environments is a burgeoning issue worldwide. Further research into the plethora of other bioactive compounds present in wastewater is imperative to ensure that effective risk management can occur, and to mitigate the impacts of human populations on natural ecosystems.

"An understanding of the natural world and what's in it is a source of not only great curiosity but great fulfilment."

- David Attenborough

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Appendices

Appendix 1: A sampling timetable example

Bromley WWTP: Retention time 5 hours.

Influent				Effluent			
Start/finish time	Bottle number	Volume (ml)	Total Volume (mL)	Start/finish time	Bottle number	Volume	Total Volume
8:00	1	250	250	13:00	1	250	250
8:30	1	500	500	13:30	1	500	500
9:00	1	750	750	14:00	1	750	750
9:30	2	250	250	14:30	2	250	250
10:00	2	500	500	15:00	2	500	500
10:30	2	750	750	15:30	2	750	750
11:00	3	250	1000	16:00	3	250	1000
11:30	3	500	1250	16:30	3	500	1250
12:00	3	750	1500	17:00	3	750	1500
12:30	4	250	1750	17:30	4	250	1750
13:00	4	500	2000	18:00	4	500	2000
13:30	4	750	2250	18:30	4	750	2250
14:00	5	250	2500	19:00	5	250	2500
14:30	5	500	2750	19:30	5	500	2750
15:00	5	750	3000	20:00	5	750	3000
15:30	6	250	3250	20:30	6	250	3250
16:00	6	500	3500	21:00	6	500	3500
16:30	6	750	3750	21:30	6	750	3750
17:00	7	250	4000	22:00	7	250	4000
17:30	7	500	4250	22:30	7	500	4250
18:00	7	750	4500	23:00	7	750	4500
18:30	8	250	4750	23:30	8	250	4750

19:00	8	500	5000	0:00	8	500	5000
19:30	8	750	5250	0:30	8	750	5250
20:00	9	250	5500	1:00	9	250	5500
20:30	9	500	5750	1:30	9	500	5750
21:00	9	750	6000	2:00	9	750	6000
21:30	10	250	6250	2:30	10	250	6250
22:00	10	500	6500	3:00	10	500	6500
22:30	10	750	6750	3:30	10	750	6750
23:00	11	250	7000	4:00	11	250	7000
23:30	11	500	7250	4:30	11	500	7250
0:00	11	750	7500	5:00	11	750	7500
0:30	12	250	7750	5:30	12	250	7750
1:00	12	500	8000	6:00	12	500	8000
1:30	12	750	8250	6:30	12	750	8250
2:00	13	250	8500	7:00	13	250	8500
2:30	13	500	8750	7:30	13	500	8750
3:00	13	750	9000	8:00	13	750	9000
3:30	14	250	9250	8:30	14	250	9250
4:00	14	500	9500	9:00	14	500	9500
4:30	14	750	9750	9:30	14	750	9750
5:00	15	250	10000	10:00	15	250	10000
5:30	15	500	10250	10:30	15	500	10250
6:00	15	750	10500	11:00	15	750	10500
6:30	16	250	10750	11:30	16	250	10750
7:00	16	500	11000	12:00	16	500	11000
7:30	16	750	11250	12:30	16	750	11250

Appendix 2: Quality control and assurance data

Analytical quality control data for each batch (ng/L).

Analyte	Batch 1				Batch 2				Batch 3			
	Blank	Sample spike	Cart Spike	Cart blank	Blank	Sample spike	Cart Spike	Cart blank	Blank	Sample spike	Cart Spike	Cart blank
15-F_{2t}-IsoP	ND	49.1	52.9	ND	ND	27.1	60.1	ND	LOD	113.7	81.3	ND
D_4-15-F_{2t}-IsoP	36.1	53.4	51.2	56.9	64.5	30.2	56.6	56.9	76.1	80.0	81.9	100.8
R% D_4-15-F_{2t}-IsoP	28.6	42.4	40.7	45.2	55.9	26.2	49.1	49.4	59.7	62.7	64.3	79.0
R_C 15-F_{2t}-IsoP	ND	115.67	130.11	ND	ND	103.7	122.2	ND	LOD	181.3	126.5	ND

Cart = Extraction cartridge